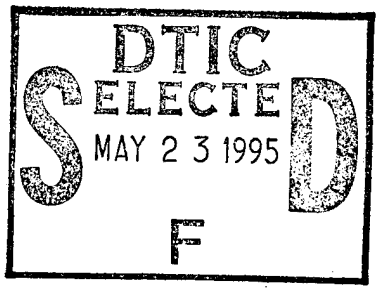
THE FLORIDA STATE UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

BIODEGRADATION OF CREOSOTE AND PENTACHLOROPHENOL USING SIMULATED LAND FARMING TECHNIQUES



By

TIMOTHY D. HODGE

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The members of the Committee approve the thesis of Timothy D. Hodge defended on April 6, 1995.

David L. Balkwill
Professor Directing Thesis

Thomas M. Roberts
Committee Member

Robert H. Reeves
Committee Member

Approved:

Joseph Travis, Chairman, Department of Biological Science

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Abstract

The objective of this project was to evaluate five land farming treatments, to determine which would best remediate creosote and pentachlorophenol contamination at and around a wood preservation treatment facility, and to determine the feasibility of applying such a treatment. These treatments were: (i) addition of water, (ii) addition of white rot fungus (*Phanaerochete chrysosporium*) and water, (iii) addition of an inoculum of bacterial isolates and water, (iv) addition of a soil inoculum from a previously remediated facility and water, and (v) addition of sodium nitrate and water.

The soil that was investigated came from a facility in Camilla, Georgia. This site had been used to impregnate a variety of wood products with creosote and (PCP). The soil was so highly contaminated that only a minimal number of microorganisms could be cultured from the samples that were on hand. It is likely that those organisms which were viable evolved some ability to metabolize these pollutants.

Based on the results of twenty-three weeks of simulated land farming, there is an indication that land farming has the capacity to degrade PCP and a number of the toxic components of creosote. Simply turning the hydrated soil showed a marked decrease in many of the compounds, though the addition of other agents showed slightly better rates of degradation. The best overall process involved the use of an inoculum of bacteria that were cultured from an enrichment medium containing creosote. In situ land farming for the remediation of creosote warrants further investigation, though it is unlikely that land farming alone will effectively remediate the contaminated soil. Instead, research may find that land farming may be used to supplement other techniques.

Chapter 1

Introduction

Treatment of Wood with Organic Preservatives

Nearly all of the wood products produced around the world are treated in one way or another with chemical preservatives to prevent (or, at least, very much retard) rotting. Although it is sometimes effective against weathering, chemical preservation is aimed primarily at preventing biological degradation of wood by microorganisms, wood-boring insects, worms, etc. Prompt treatment with preservatives is more critical in tropical areas, but wood from temperate regions is also susceptible to biological damage. Depending on the circumstances, wood might be treated when timber is first harvested, while it is being stored, and/or after each reshaping (*i.e.*, cutting) step. The extent and type of treatment depends on the wood's intended purpose. Protection from biological attack is especially important for wood products that remain in contact with soil or water during use. Commercially important examples of these products include railroad ties, telephone poles, fence posts, and marine and freshwater pilings.

Several approaches are commonly used to preserve wood products with chemicals. Some of these approaches make use of inorganic compounds, whereas others are based on the use of organic chemicals. The two most frequently used organic compounds for wood preservation in situations where a high degree of protection is required are coal tar creosote and pentachlorophenol (PCP). World wide, there are 3,000 to 3,500 wood treatment facilities that consume 350,000 metric tons of creosote and 110 million liters of organic solvent type preservatives, including PCP (UNEP, 1994). The United States has

approximately 587 wood treatment facilities and produces 500 million cubic feet of treated wood products annually. Of this total production, 97.8 million cubic feet are treated with creosote and 48.6 million cubic feet are treated with PCP (EPA, 1991a). One hundred or more additional treatment sites in the United State are no longer operational (Mueller et al., 1989a) but can serve as sources of environmental contamination (see below).

Coal Tar Creosote

Creosote, a complex coal tar distillate, has been used as a wood preservative around the world for over 150 years (UNEP, 1994). It has proven to be a very effective insecticide, fungicide, and bactericide. It has also been shown to add a degree of physical durability to treated wood.

Creosote is composed of over 100 different chemicals, many of which are carcinogenic and/or teratogenic. The exact composition and molecular weight vary from batch to batch because of impurities and differences in the exact chemical composition of the coal tars from which creosote is made. A typical batch of creosote is composed of approximately 85% polynucleated aromatic hydrocarbons (PAHs); 10% phenolic compounds; and 5% N-, S-, and O-heterocyclics (Mueller et al., 1989b). Most of the PAHs in creosote are unsubstituted and, therefore, relatively resistant to biological degradation. The major specific components of coal tar creosote and some of their characteristics are listed in Tables 1-1 through 1-3. Creosote-treated wood often contains additional compounds because the creosote is usually mixed with petroleum products prior to use.

Pentachlorophenol (PCP)

Pentachlorophenol is a man-made product that occurs as colorless, needle-like crystals in its pure form. When it contains impurities, PCP appears as dark-gray-to-brown dust, beads, or flakes. It has a characteristic sharp smell when hot but is essentially odorless at room temperature. PCP typically occurs in two forms: (i) as a sodium salt

Table 1-1. Predominant polycyclic aromatic hydrocarbons in coal tar creosote[a].

Compound	Percent of total PAH (wt)	Molecular weight	Aqueous solubility (mg/L, 25°C)
Naphthalene	13	128.2	31.7
2-Methylnaphthalene	13	142.2	25.4
Phenanthrene	13	178.2	1.3
Anthracene	13	178.2	0.07
1-Methylnaphthalene	8	142.2	28.5
Biphenyl	8	154.2	7.5
Fluorene	8	166.2	2.0
2,3-Dimethylnaphthalene	4	156.2	3.0
2,6-Dimethylnaphthalene	5	156.2	2.0
Acenaphthene	4	154.2	3.9
Fluoranthene	4	202.3	0.26
Chrysene	2	228.2	0.002
Pyrene	2	202.3	0.14
Anthraquinone	1	208.2	-
2-Methylanthracene	1	192.3	0.04
2,3-Benzo(b)fluorene	1	216.3	0.002
Benzo(a)pyrene	1	252.3	0.003

[a]Source: Mueller et al, 1989b.

Table 1-2. Predominant phenolic compounds in coal tar creosote[a].

Compound	Percent of total phenolics (wt)	Molecular weight	Aqueous solubility (mg/L)
Phenol	20	94.1	82,000 (15°C)
o-Cresol	10	108.1	25,920 (25°C)
m-Cresol	10	108.1	23,500 (20°C)
p-Cresol	10	108.1	24,000 (40°C)
Pentachlorophenol	10	266.4	14 (20°C)
2,5-Xylenol	7.5	122.2	3544 (25°C)
3,5-Xylenol	7.5	122.2	4888 (25°C)
2,3-Xylenol	5	122.2	4570 (25°C)
2,4-Xylenol	5	122.2	6232 (25°C)
2,6-Xylenol	5	122.2	6049 (25°C)
3,4-Xylenol	5	122.2	4766 (25°C)
2,3,5-Trimethylphenol	5	136.3	-

[a]Source: Mueller et al., 1989b.

Table 1-3. Predominant heterocyclic compounds in coal tar creosote[a].

Compound	Percent of total heterocyclics (mg/L)	Molecular weight	Aqueous solubility (mg/L)
N-Heterocyclics and N-containing aromatics:			
Quinoline	10	129.2	6718 (20°C)
Isoquinoline	10	129.2	4522 (20°C)
Carbazole	10	167.2	1 (20°C)
2,4-Dimethylpyridine	10	107.2	-
Acridine	5	179.2	5 (20°C)
Aniline	5	93.1	3400 (25°C)
2-Methylquinoline	5	143.2	-
4-Methylquinoline	5	143.2	-
Pyrrole	5	67.1	-
Pyrrolidine	5	71.2	-
S-Heterocyclics:			
Benzo(b)thiophene	10	134.2	130 (20°C)
Dibenzothiophene	10	184.3	2 (24°C)
O-Heterocyclics:			
Dibenzofuran	10	168.2	10 (25°C)

[a]Source: Mueller et al., 1989b.

that is soluble in water (ADSDR, 1989), and (ii) as pure PCP with a molecular weight of 266.4 (Mueller et al., 1989b). Like creosote, PCP is mixed with petroleum products prior to use as a wood preservative. Used crankcase oil is sometimes used for this purpose, simply to ensure that the wood looks as if it has been treated.

Wood Treatment and Environmental Contamination

Creosote and PCP pose little danger to humans when they are properly controlled. Unfortunately, they are often released into the environment because the proper controls are lacking. Many wood treatment facilities are constructed in ways that allow freshly treated wood to drip onto the ground. Concentrated preservative solutions are sometimes spilled directly onto the ground by careless handling and/or defective equipment. Over a period of time, wood-treating compounds may also leach off treated products that have been stockpiled. If the contamination from these sources were cleaned up quickly, there would be little or no environmental impact. When such sources are allowed to discharge onto the soil for extended periods of time (as is often the case), however, the contaminants are likely to migrate through the soil into the ground water. Ground water serves as the primary source of fresh water for drinking and irrigation in the United States. Therefore, the movement of carcinogens and/or teratogens like PCP and creosote into ground water creates a significant hazard to public health. Movement of wood-treating compounds into and through the subsurface can be quite slow, which simply means that contamination problems may persist for many years after a wood-treating site is closed. Creosote has been found at 31 of the 1,177 sites that are on EPA's National Priorities List (NPL). This number could increase as more and more sites are tested for the presence of creosote (ATSDR, 1990). PCP has been found at 84 of the NPL sites (ATSDR, 1989). This number could also increase as more sites are characterized.

The oral LD_{50} in rats for creosote is 725 mg/kg body weight, while the oral LD_{50} for PCP is 27 mg/kg body weight in rats. No LC_{50} data are available for creosote, but fish have a 96-hour LC_{50} of 60 to 600 μg/L when exposed to PCP. Occupational airborne exposure limits for creosote in the United States are 0.2 mg/m^3. In Switzerland, the exposure limit for PCP is 0.5 mg/m^3 (UNEP, 1994). There are no carcinogenicity data available on creosote per se. However, many of the constituents of creosote have strong links to cancer. Benzo(a)pyrene, for example, is a strong carcinogen that affects most organs and tissues. Data for PCP also suggest a cancer link (Sax, 1981). Chronic PCP exposure levels as low as 20 ppm in food have been shown to cause liver damage in rats over an eight-month period. Workers exposed to airborne PCP (0.99 ppm) suffered eye and nose irritation (STSDR, 1989).

Methods for Remediation of Contaminants Associated with Wood Treatment Physical Methods

Several alternative methodologies are now available for removal of compounds like creosote and PCP from a contaminated site. Perhaps the simplest of these is to dig up the contaminated soils and subsoils and move them to another location. In most cases, the contaminated materials are then placed in special clay- and/or plastic-lined pits designed to prevent seepage of contaminants into the subsurface at the new site. This approach serves to remove the source of contamination (or, at least, most of it) from a contaminated site, but it does little or nothing to eliminate the contaminants themselves.

Another approach for remediation of contaminated wood-treating sites involves digging up the soils and subsoils, trucking them to a specialized incineration facility, and incinerating them at high temperature to "burn off" the contaminants. This approach is very expensive because the soil must be trucked by a licensed hazardous waste hauler (at rates far higher than those for trucking nonhazardous materials), usually over considerable

distances. (Long hauls are required frequently because only a few states issue permits for incineration of contaminated soils at this time.) Moreover, there is always some chance that contaminants will be transferred to the atmosphere, even though the high-temperature incinerators are designed to minimize this problem. Another drawback of incineration is that it destroys the natural organic components of the soil along with the contaminants, thereby producing an infertile "soil-like substance" that must be mixed with organic material in order to support plant life. As a result, there is little or no commercial demand for the final incineration product.

Bioremediation

Bioremediation is the use of living organisms (usually microorganisms) to remove toxic substances from contaminated environments. This approach has gained increased popularity in recent years because it is often less costly and more effective than physical methods for removal or containment of contaminants. A variety of different technologies for bioremediation are now available, and several of these have been evaluated at wood-treating sites in the United States.

Land farming is probably the simplest method for bioremediation of contaminated soils. In this approach, the soil is dug up and spread out over an appropriate area (usually above a layer of packed clay to prevent downward migration of contaminants) and tilled periodically with standard farm equipment. Tilling aerates the soil (EPA, 1991b), which greatly stimulates microbial degradative activity. A limited amount of anaerobic degradation of contaminants has been observed in reduced environments, but most of the known metabolic pathways by which organics are degraded require oxygen. Moreover, aerobic degradation is more likely to lead to full mineralization of the contaminants (*i.e.*, oxidation to CO_2), whereas anaerobic degradation is more likely to produce unwanted, partially degraded organics. Appropriate moisture levels must be maintained to optimize degradative activity; this can be done by periodic irrigation if local rainfall is inadequate.

In addition to oxygen and water, microbial communities in soil being land farmed might require inorganic nutrients such as nitrogen or phosphorous. This can be supplied by mixing in appropriate solid fertilizers while tilling the soil or by dissolving the required compounds in water used for irrigation.

Land farming is relatively inexpensive, and it sometimes works very effectively. For example, a site with creosote-contaminated soil in Wilmington, North Carolina was land-farmed for several years, after which contaminant levels were so low that the site could be turned into a public park. On the other hand, land farming is a comparatively slow method and (as was the case in Wilmington) often requires several years when used to remediate relatively complex and recalcitrant organic contaminants like creosote. During this time, abiotic factors such as photodegradation and volatilization may play a role in degradation. Many organic compounds are light-sensitive and can be degraded or partially degraded by electromagnetic energy. Many toxic organics are also at least somewhat volatile and can dissipate directly into the air. The rate at which this happens depends on how volatile the compounds actually are and varies over a wide range. When the potential for it is high, volatilization is a significant disadvantage of land farming because it decreases air quality. Because of this, land farming may not be permitted in areas where air quality is already poor unless it can be demonstrated that biological activity causes most of the degradation, rather than volatilization or photodegradation.

Mueller et al. (1991a, b) have explored a different approach for bioremediation of wood treatment contaminants: the use of a bioreactor to treat contaminated ground water and slurries of contaminated soil. Bioreactors typically contain some type of solid matrix that provides a surface to which microorganisms can attach and develop into a biofilm. As the contaminated materials pass through this matrix, the microorganisms in the biofilm use a variety of extracellular and intracellular enzymes to degrade the organic contaminants as their sources of energy and carbon. Mueller et al. (1991a) converted creosote- and PCP-

contaminated soils and subsurface sediments to slurries and then processed them through a bioreactor. They found that contaminants in slurried sediments were degraded somewhat more rapidly than those in slurried soils. In a separate study, Mueller et al. (1991b) found that bioreactor treatment of contaminated ground water from the same site was also quite effective. Up to 100% of the lower-molecular-weight PAHs were removed by the indigenous microbial populations. (The bioreactor was not deliberately inoculated with microorganisms; rather, the native microorganisms in the contaminated water were allowed to establish a biofilm.) Unfortunately, the final effluent from the bioreactor did not meet drinking water standards because the high-molecular-weight PAHs were not removed by this approach.

Another possible approach for the bioremediation of wood treatment contaminants would be to deliberately inoculate contaminated soil or water with microorganisms from a site where the same types of contaminants have existed for a considerable period of time. This approach is based on the idea that the contaminants themselves will, over a period of time, bring about a natural enrichment for microbial species that can degrade them. Any species that can degrade (or, through mutation, acquires the ability to degrade) one of the contaminants has a carbon and energy source that is not available to the other members of the microbial community. As a result, that species has a competitive edge over the others and grows faster, eventually becoming the numerically predominant form in the community. There is some evidence to indicate that this sort of natural enrichment actually occurs in the case of wood treating contaminants. Thomas et al. (1989) reported that the microflora near the contamination plume of a creosote treatment facility had the ability to mineralize some of the chemical constituents of creosote, whereas the microorganisms in uncontaminated soil from that area lacked this ability. Similarly, Mueller et al. (1989a) found that bacteria enriched from soil that came from a former creosote waste water evaporation pond (using an enrichment that contained fluoranthene

as the sole carbon source) were effective at degrading high-molecular-weight PAHs. These experiments suggested that, given the correct level of exposure, certain soil microorganisms could activate pre-existing genes for degradation of creosote components. It is also possible that the organisms could evolve novel genes that would enable them to cope with a contaminant and, eventually, use it as a nutrient source. This would be more conceivable if the system were nutrient limiting and the microorganisms had to incorporate alternate energy sources into their metabolic pathways (Gibson, 1984).

In general, the literature on this subject suggests that creosote and PCP can be bioremediated, despite their toxicity and environmental persistence. There is little or no agreement, however, on how bioremediation would best be accomplished. It is entirely possible that different sites would be remediated most effectively by different techniques. In any case, the U. S. Environmental Protection Agency (EPA) acknowledges that in situ bioremediation procedures like land farming may require a long time to complete. Based on their guidebook for citizens, they appear willing to accept a relatively slow process if it is effective. Remediation of a typical contaminated site by traditional physical methods like high-temperature incineration could cost $140 million, compared to only $40 to $50 million for bioremediation by land farming (EPA, 1991b). The savings may well be worth the wait in a situation where rapid cleanup is not required.

Mechanisms for Biodegradation of Wood Treating Chemicals

Most of the chemical components of creosote are insoluble in water and, thus, are somewhat inaccessible to microorganisms. However, some species secrete extracellular enzymes that render the potential substrates more hydrophilic, thereby making it easier to solubilize them (Gibson, 1984).

The polycyclic nature of many creosote components makes them very difficult to degrade. Full mineralization of such compounds often depends on the collective efforts of

a microbial consortium in which one species partially degrades the initial substrate, another species partially degrades the breakdown product of the first species, etc. Some complex organic compounds (such as PAHs) do not yield much energy when they are degraded or partially degraded by microorganisms. As a result, the microorganisms can only degrade the low-energy compound when a second compound that yields more energy (typically a much simpler organic) is available in the environment.

The general metabolic pathway for bacterial degradation of PAHs begins with the use of a dioxygenase to produce a *cis*-dihydrodiol from the PAH. This *cis*-dihydrodiol is then dehydrogenated to a catechol, and the catechol subsequently undergoes ring fission (Higson, 1991). The entire ring structure may be broken down by going through several cycles of this pathway. Benzene, a much simpler organic compound, can be used to demonstrate a single pass through this pathway (Figure 1-1).

Microbial degradation of PCP usually occurs by one of three metabolic processes: dechlorination, methylation, or oxidation. Dechlorination of chlorophenols results in a decrease in toxicity because of a decrease in hydrophobicity and reactivity (Bryant and Shultz, 1994). Methylation of chlorophenols, which typically occurs under anaerobic

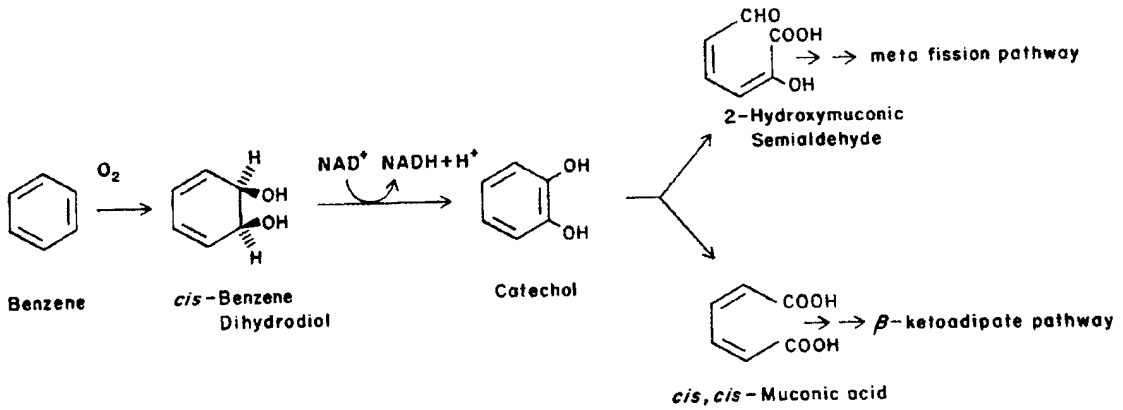

Figure 1-1. A proposed metabolic pathway for the degradation of benzene (Gibson, 1984).

conditions, leads to decreased toxicity because of a decrease in reactivity (Rochkind-Dubinsky, 1987). Oxidation of chlorophenols leads to enhanced toxicity owing to increased reactivity, despite the decreased hydrophobicity (Bryant and Shultz, 1994). It seems that oxidation and hydroxylation of PCP are short-lived intermediate steps (Rochkind-Dubinsky, 1987). Figure 1-2 shows a proposed pathway for the degradation of PCP. The potential for an increase in toxicity can be a significant drawback of bioremediation if it is applied to chlorinated compounds like PCP.

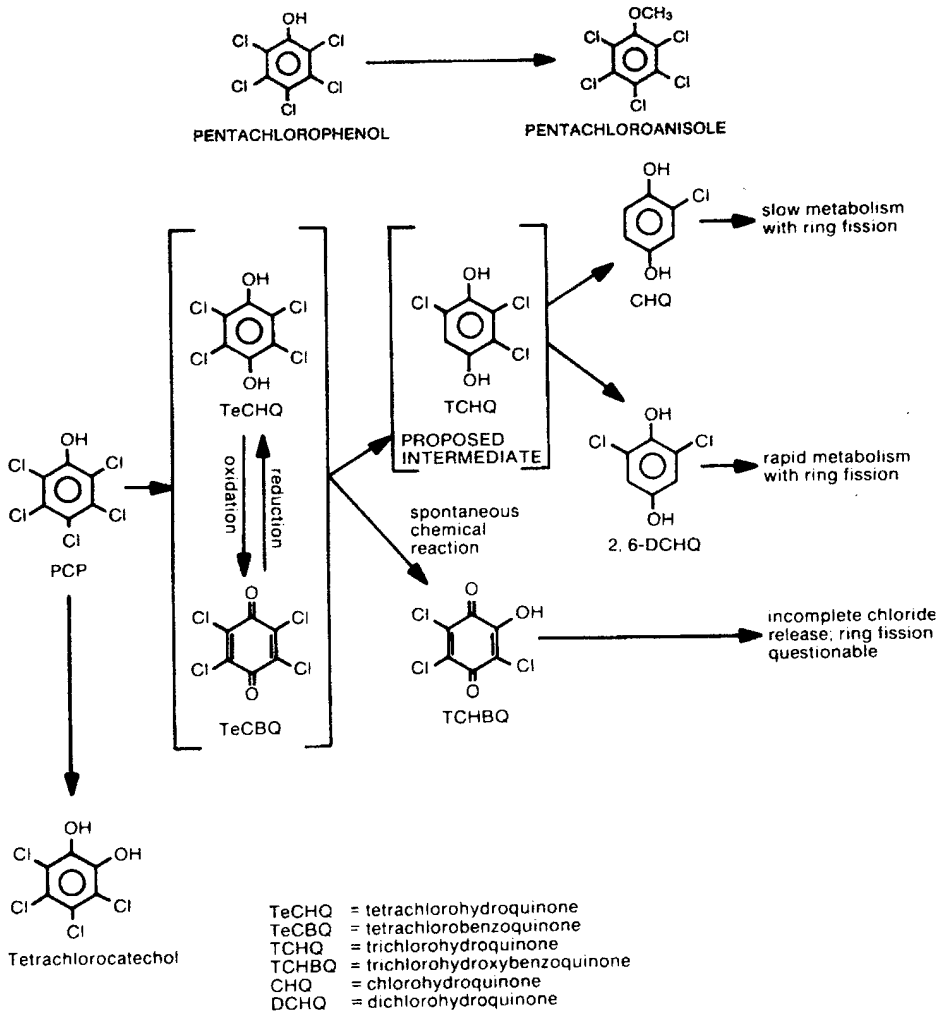

Figure 1-2. Proposed metabolic pathways for the degradation of pentachlorophenol (PCP) (Rochkind-Dubinsky, 1987).

The White Rot Fungus

Phanerochaete chrysosporium, more commonly known as the white rot fungus, can degrade a wide variety of complex organic compounds. This capability results from the enzymes that the fungus uses to degrade lignin, the principal structural component of woody plants. Lignin is essentially a "three-dimensional aromatic polymer that contains a variety of stable carbon-carbon and ether linkages between monomeric phenylpropane units" (Higson, 1991). To decompose lignin, the white rot fungus utilizes ligninases (which are peroxidases), along with hydrogen peroxide and veratryl alcohol. Peroxide, veratryl alcohol, and the enzymes all function as extracellular agents that produce free radicals and synergistically oxidize lignin, as well as other aromatic compounds (Shah et al., 1992). Once the polymer has been broken down into a more soluble substrate, the fungus can assimilate it through the Krebs cycle. A distinct advantage to this system of degradation is that the process is non-specific. Therefore, the fungus would not have to have a prior exposure to the toxic substance to be able to degrade it (Higson, 1991, Bumpus, 1985).

There is strong evidence that white rot fungus has to capacity to mineralize a variety of halogenated organic compounds. Though the fungus could not mineralize compounds that are saturated with chloride, such as mirex, it could convert them to other chemicals. Less saturated compounds such as lindane and chlordane were biodegraded extensively by white rot fungus (Kennedy, 1990). PCP falls into this latter category.

Degradation of PCP seems to work better in a nitrogen poor environment, though white rot fungus has been observed to substantially biodegrade PCP in a nitrogen-sufficient environment. This suggests that there are other degradative systems employed by the fungus, in addition to the ligninases (Mileski, 1988). Shah et al. (1992) proposed four degradation mechanisms for the lignin peroxidases of white rot fungus. He suggested

that direct oxidation and co-oxidation were pathways for degradation of compounds such as cyanide, EDTA and oxalate (which are good electron donors). For electron acceptors, such as PCP, a process of oxidation by hydroxyl radicals or a reduction in the presence of an electron donor are two likely routes.

Objective of the Present Study

The objective of the present study was to evaluate the feasibility of five relatively inexpensive and simple approaches for bioremediation of soils contaminated with creosote and PCP. The five possible approaches were evaluated by carrying out treatability studies in laboratory-scale "macrocosms" that contained approximately 900 g of contaminated soil as a substrate for microbial degradation. Degradation of the contaminants was monitored by chemical analysis of the soils in the macrocosms (to determine the concentrations of PCP and selected creosote components) after 10 and 23 weeks of treatment. The five treatments that were evaluated were as follows:

Treatment 1: The soil was periodically aerated and moistened. This treatment was designed to mimic simple land farming (see above) without the addition of nutrients.

Treatment 2: The soil was inoculated with the white rot fungus and periodically aerated and moistened. This treatment was designed to determine whether addition of the white rot fungus yielded a significant improvement over land farming (*i.e.*, treatment 1) in terms of the extent to which contaminants were degraded.

Treatment 3: The soil was inoculated with a mixture of bacteria that had been isolated from enrichment cultures containing creosote as their only source of carbon, then periodically aerated and moistened. The rationale for this treatment was that enrichment culture isolates would be able to degrade at least some creosote components and should, therefore, be able to accelerate the removal of contaminants. For such an approach to be economically feasible, of course, any improvement that was seen over simple land farming

would have to be large enough to justify the cost of applying the bacterial inoculum on a field scale.

Treatment 4: The soil was "inoculated" with a small quantity of soil that was previously contaminated with creosote, then periodically aerated and moistened. The inoculum soil, which was reasonably free of contaminants when used in this study, came from a site in Delaware at which land farming had been carried out for several years to bioremediate creosote and other wood treating chemicals. The reasoning behind this treatment was that the land-farmed soil ought to contain microorganisms that can degrade creosote because a natural enrichment for such organisms must have occurred while the soil was being bioremediated. The goal, then, was to determine whether a soil like this could be used as an inoculum in place of a pure inoculum of cultured bacteria, like the one used in treatment 3. If this approach worked very effectively, it might actually create a commercial value for soils from land farming operations.

Treatment 5: Sodium nitrate was added to the soil, after which it was aerated and moistened periodically. Several bioremediation studies have reported that degradation was enhanced by adding nitrogen to the material being treated, apparently because it was the limiting nutrient for microbial growth. The purpose of this treatment, then, was simply to see if nitrate addition had any significant effect on degradation of creosote and/or PCP in the soil that was examined in this study.

All treatments were evaluated with respect to the number of creosote components that were degraded and the extent to which each was degraded. The results are presented here, along with conclusions regarding the feasibility of relatively simple bioremediation approaches as a methodology for cleaning up soils that are contaminated with creosote and other chemicals used in the wood treating industry.

Chapter 2

Materials and Methods

Procurement of Soil Samples

The soil samples used in this study are listed in Table 2-1. Four of these samples (CB10, CB11, CB12, and CB13) were collected from a wood treating facility in Camilla, GA, shortly before this facility was closed and placed on the Super Fund List by the EPA. These samples were collected from near the surface in heavily contaminated areas, using a regular post hole digger. The samples were then stored at room temperature (20-25°C) in plastic Ziploc™ bags (DowBrands, Indianapolis, IN).

One soil sample (CB02) was obtained at the site of a former wood treating facility in Wilmington, NC. The soil at this site was heavily contaminated with creosote and other wood treating chemicals when the treatment plant closed, but it had been land farmed (see Literature Review) for several years when the sample was collected. By then, the soil was reasonably free of contaminants. (In fact, the site was turned into a public park about one month after the sample was acquired.) This soil was considered likely to contain bacteria that are capable of degrading creosote components because a natural enrichment for such bacteria should have occurred during land farming. The sample was air dried and stored at room temperature in a Ziploc™ bag until it could be used in this study.

Three soil samples (A, B, and C) were collected at a site upstream (and unlikely to be influenced by) the former wood treating facility in Camilla, GA. The collection site was in a farm field that had not been worked for at least three years. The farm was located 1.5 miles NE of Camilla, on the west side of Highway 112. Three samples were taken (at the

Table 2-1. Soil samples used in this study.

Sample name	Sample site	Site characteristics	Sampling method[a]	Sample size (g)
CB10	Camilla, GA	Heavily contaminated wood treating facility; waste lagoon	PD	1,145
CB11	Camilla, GA	Heavily contaminated wood treating facility; waste lagoon	PD	980
CB12	Camilla, GA	Heavily contaminated wood treating facility; soil subject to drippage from pressure treatment equipment	PD	1,044
CB13	Camilla, GA	Heavily contaminated wood treating facility; waste lagoon	PD	1,072
CB02	Wilmington, NC	Previously contaminated wood treating facility; soil was land-farmed for several years (thought to be clean)	SS	350
A	Camilla, GA	Farm field (unworked for 3 years)	SS	5,000
B	Camilla, GA	Farm field (unworked for 3 years)	SS	5,000
C	Camilla, GA	Farm field (unworked for 3 years)	SS	5,000

[a]PH = post hole digger; SS = shovel sterilized by flame or disinfected with chlorine bleach

corners of triangle that was approximately 10 m on each side) with a shovel that had been disinfected with chlorine laundry bleach. The sparse vegetation and detritus were removed from the top of each sample site (to a depth of 3 to 5 cm) prior to collection of the sample itself. The samples were then stored at 4°C in Ziploc™ bags until they were used in this study.

Enumeration of Culturable Microorganisms

The numbers of culturable microorganisms in original soil samples (above) and soil treatability study samples (below) were determined by the plate count technique. Samples were prepared for plate counting by blending in 0.1% $Na_4P_2O_7 \cdot 10H_2O$ at pH 7.0 (Balkwill and Ghiorse, 1985). Serial ten-fold dilutions of the blended samples were then prepared in phosphate-buffered saline solution (PBS; Appendix A) and spread-plated (in triplicate) on 5% Trypticase Soy Agar (5% TSA; Appendix A). All plates were incubated aerobically at 25°C for two weeks, after which the number of microbial colonies that developed on each plate was counted and recorded. The total number of culturable microorganisms was then calculated by multiplying the average number of colonies on the plates for the "countable" dilution (*i.e.*, the dilution that produced an average of 30 to 300 colonies per plate; Claus, 1988) by the dilution factor. The results were recorded as the number of colony-forming units (CFU) per gram of original sample (wet wt).

Isolation and Characterization of Microbial Strains

Microbial strains were isolated from selected colonies appearing on the plates for plate counts (above) and enrichment cultures (below) by streaking (and, when, necessary, restreaking) on fresh plates of 5% TSA until all resulting colonies appeared to be uniform in appearance. In all cases, plates were incubated aerobically at 25°C until colonies were sufficiently developed to examine their morphological traits.

The cell morphological traits of each isolate were determined by Gram-staining of cells grown in 5% Trypticase Soy Broth (TSB) on a rotary shaker at 25°C. Gram stains were performed as described by Claus (1988). The resulting slides were examined with a Zeiss bright-field microscope, using a 100X oil-immersion objective lens. Cell shape and reaction to the Gram stain (positive or negative) were recorded in each case.

The colony morphological traits of each isolated microbial strain were determined by careful examination of well-separated colonies on 5% TSA plates as described by Smibert and Krieg (1981). The following colony morphological traits were recorded: color, type of surface, opacity, texture, type of edge, elevation, and any unusual or highly distinctive features (such as tendency to spread over the surface of the plate or morphological traits that are characteristic of streptomycetes or other specific groups of bacteria). All of the colony morphological traits were entered (in abbreviated form) into a digitized data base, using standard software for microcomputers (Microsoft Excel for Windows, Version 3.0; Microsoft Corp., Redmond, WA), so that they could be sorted or otherwise analyzed in the future.

Selected physiological characteristics of some isolates were determined with API Rapid NFT test kits (bioMérieux Vitek, Inc., Hazelwood, MO). These Rapid NFT kits test for 21 physiological traits (9 specific enzymatic capabilities and the ability to use 12 different organic compounds aerobically as sole sources of carbon; see Appendix B). The test kits were inoculated, incubated, and scored as specified by the manufacturer. Results were recorded and entered into a digitized data base as described above.

Preservation of Isolated Microbial Strains

Microbial strains isolated as described above were preserved for later use (*e.g.*, in the soil treatability study, below) or examination by freezing shortly after their isolation. In each case, cell material from a restreak plate (above) was transferred to 50 ml of TSB

and incubated at 25°C on a rotary shaker until obvious turbidity developed in the broth. The cultures were then concentrated by centrifugation, suspended in 2 ml of fresh TSB containing 7% sterile dimethylsulfoxide, and frozen at -75°C.

Enrichments for Creosote- or PCP-Degrading Microbial Isolates

A standard enrichment culture approach was used to isolate bacteria that might be capable of degrading creosote compounds. Enrichments were attempted with the following soil samples (see above): CB02, CB10, CB11, CB12, CB13, and C. In each case, 0.1 g of soil was inoculated into 100 ml of Stanier's standard mineral base medium (Appendix A) containing 30 µl of coal tar creosote (concentrated stock from the former Camilla, GA wood treating facility). The only significant source of organic carbon in this medium was the creosote. Therefore, the only microorganisms likely to grow in it (and, eventually, to replace the ones that were present initially) were those that could degrade one or more of the creosote constituents as their source of carbon and energy.

The enrichment cultures were incubated at 25°C on a rotary shaker for five weeks. Each week, a small amount of medium was removed (from each flask) with an inoculating loop and streaked for isolation on 5% TSA. The TSA plates were incubated aerobically for two weeks at 25°C, after which microbial strains were isolated from selected colonies, characterized, and preserved as described above.

By design concurrent duplicate enrichments of CB02, CB11, and C were used. CB11 did not show any growth throughout the five-week enrichment period, so this enrichment was later repeated (total of three enrichment attempts).

Source and Growth of the White Rot Fungus

A culture of *Phanaerochete chrysosporium*, commonly known as the "white rot fungus," was provided by the U. S. Dept. of Agriculture Forest Products Laboratory in Madison, WI. The fungus was initially grown in Difco Malt Broth (Difco Laboratories,

Detroit, MI) on a rotary shaker at 25°C until the broth became densely turbid. Twenty-five ml of turbid culture broth was then mixed with approximately 200 g of sterile wood chips in a 2.8-liter Fernbach flask. The Fernbach flask was incubated at 25°C for several weeks, until the fungus grew extensively and sporulated (as evidenced by the appearance of a white film over the surfaces of the wood chips). The sporulated fungus was simply stored at room temperature until it was needed for the treatability study (below).

To grow a fresh supply of the white rot fungus for the treatability study, a small amount of the spore stock (above) was inoculated into 50 ml of sterile 5% PTYG broth (Appendix A) and mixed with 500 ml of sterile wood chips in a 2.8-liter Fernbach flask. Sterile water (approximately 130 ml) was then added until the wood chips were visibly moist but not dripping wet. The inoculated wood chips were incubated at 25°C for one week, after which they were used in the treatability study as described below.

Preparation of a Bacterial Inoculum for the Treatability Study

Twenty bacterial strains that were isolated from enrichment cultures (see above) were selected for use as an inoculum in the treatability study (below), based on: (i) how long the enrichment was incubated before the bacterium was isolated (minimum of three weeks); (ii) uniqueness of the isolate, based on colony morphological traits (to avoid likely duplicates); and (iii) the amount of time required to grow the isolate to a high density in broth media (one week or less). (See Results for a detailed description of the selected isolates.)

Each of the 20 selected strains was streaked on a 5% TSA plate to check purity. The plates were incubated at 25°C until colonies developed, after which material from a single colony was transferred to 100 ml of 5% TSB. The broth cultures were incubated at 25°C on a rotary shaker for 24 hours and concentrated by centrifugation. The cell pellets for all 20 cultures were then resuspended in a few ml of sterile distilled water, combined in

a single centrifuge bottle, concentrated once again by centrifugation, and resuspended in 75 ml of tap water. The resuspended cells served as the inoculum for the treatability study (see below).

Treatability Study

The primary focus of this investigation was a soil treatability study in which five possible methods (all of which were simple and relatively inexpensive) for bioremediation of soils contaminated with creosote and PCP were tested on a laboratory scale. The soil treatments were carried out in "macrocosms" consisting of approximately 900 g of soil in brown glass jars. All treatments were carried out for a 23-week period, during which the numbers of culturable microorganisms in the soils were monitored by plate counting and the concentrations of PCP and various creosote components were monitored by GC-MS (gas chromatography-mass spectroscopy) analysis.

Preparation of the Contaminated Soil Sample

Soil samples CB10, CB11, CB12, and CB13, all of which were contaminated with creosote and PCP (above), initially consisted of rather large, compacted clumps that were hard to manipulate. Therefore, the clumps were broken down into much smaller particles (1 to 3 mm in diameter), first with a sterile spatula and, then, by hand crushing. The four crushed soils were combined and mixed thoroughly in a large plastic bin. When the mixed soils were weighed, it was found that roughly 1.5 kg of additional soil would be needed to set up and carry out the treatability study as planned. The required increase was achieved as follows. Approximately 1.5 kg of soil from sample C (which was uncontaminated; see above) was sterilized (by autoclaving it three times), dried, broken up into small particles, and mixed thoroughly with the previously mixed contaminated soils. The combined soils were then mixed again for ten minutes on each of three consecutive days. The well-mixed soil was stored at room temperature (covered with aluminum foil) until it was used in the treatability study as described below.

Soil Treatments

Three hundred grams of the fully prepared contaminated soil (above) was mixed thoroughly with 25 ml of tap water. Approximately 250 g of this mixture was analyzed by GC-MS (see below) as a "0-time" control, in order to determine the starting (*i.e.*, pre-treatment) concentrations of PCP and selected creosote components in the soil.

For each of the five soil treatments that were evaluated in this study, 900 g of the prepared contaminated soil was amended as described individually below and placed in a 32-oz QorPak™ brown glass jar fitted with a Teflon-lined screw-cap lid (Fisher Scientific, Orlando, FL). In all cases, it was necessary to pack the soil lightly by shaking it down as the jars were filled.

Treatment 1. The soil was mixed thoroughly with 75 ml of tap water.

Treatment 2. The soil was mixed thoroughly with 75 ml of tap water and 50 g of wood chips inoculated with white rot fungus as described above.

Treatment 3. The soil was mixed thoroughly with 75 ml of the bacterial inoculum that was prepared with enrichment-culture isolates as described above.

Treatment 4. The soil was mixed thoroughly with 50 g of soil sample CB02 (see above) and 79.2 ml of tap water.

Treatment 5. The soil was mixed thoroughly with 75 ml of tap water containing 1 g of sodium nitrate.

The lids on all five jars were closed tightly and then backed off one complete turn. The jars were then incubated at 25°C for 23 weeks as described below.

Handling of Soil Treatments during the Treatability Study

The soils for each treatment were aerated every two days during the first 10 weeks of the treatability study, in order to avoid the development of anaerobic conditions. After the first samples were removed for GC-MS analysis (below), the soils were aerated every three days. (Anaerobic conditions were then less likely to develop because removal of the

first analytical sample increased the head space in the jars.) To aerate the soils, they were poured into an aluminum baking pan (23 x 32 x 5 cm) and stirred for several minutes with a metal spatula. (Both the pan and the spatula were disinfected with Lysol™.) The soils were then returned to their jars.

Small quantities of tap water were periodically added to (and mixed with) each of the five treatment soils during the 23-week study, in order to maintain moisture levels that would support microbial growth and metabolic activity. The amounts of water added and the days on which they were added were as follows: 5 ml on day 11, 5 ml on day 27, 5 ml on day 37, 5 ml on day 47, 4 ml day 72, 3 ml on day 89, and 4 ml on day 115 (total of 31 ml over 115 days).

Chemical Analysis for PCP and Creosote Components

Samples of treatment soils were analyzed for PCP and creosote components after 10 and 23 weeks of incubation under the conditions detailed above. Approximately 250 g of soil was analyzed in each case. All analyses were performed by Savannah Laboratories and Environmental Services, Inc. (Tallahassee, FL), an EPA- and State of Florida-certified analytical laboratory. The concentrations of PCP and 17 selected components of creosote (see Results and Appendix C) were determined by GC-MS analysis, as specified in EPA Method SW-846 (also known as EPA 8270 - "Semivolatile Organics").

Chapter 3

Results

Soil Characteristics

Soil sample CB02, which came from the Wilmington, NC site, was dark brown in color. It consisted mostly of small detritus particles, a small fraction of sand, and clay. The soil was dry and compacted. Its aroma was similar to that of normal, uncontaminated soil. An initial plate count for enumeration of viable microorganisms indicated that this soil contained 9.8×10^5 CFU/g (wet wt).

Soil samples CB10, CB11, CB12 and CB13, which came from contaminated areas at the Camilla, GA wood-treating facility, were grayish black in color. Samples CB10 and CB13 were slightly lighter in color than the other two. Each sample consisted mostly of sand and clay, with a few small (10 mm long) wood fragments. The soils were compacted and fairly dry. Their aroma had a slight hint of creosote (a naphthalene-like odor). An initial plate count showed that CB10 contained 1.5×10^5 CFU/g (wet wt). In contrast, the count for sample CB11 was below the detection limit of the plating procedure (~100 CFU/g). Samples CB12 and CB13 were not enumerated.

Soil samples A, B, and C, which came from a farm field Northeast of Camilla, GA, were a rich dark brown in color. Each sample consisted of sand and clay, with a large amount of detritus. Root hairs ran throughout the samples. The soils were moist and loosely compacted. Their aroma was strong but similar to that of normal, uncontaminated soil. An initial plate count indicated that sample C contained 4.5×10^6 CFU/g (wet wt). Samples A and B were not enumerated.

Enrichment Culture Experiments

Turbidity, which usually indicates that microbial growth is occurring, developed within three days after soil sample CB02 was inoculated into an enrichment medium that contained creosote as a sole source of carbon (see Materials and Methods). The medium (initially almost transparent) turned dark brown within one week and remained that color throughout the rest of the five-week incubation period. The creosote aroma decreased noticeably within three weeks and was very weak after five weeks. The majority of the colonies selected from CB02 were cream, yellow, or clear. In addition to these basic colors, a pink colony was selected from week four, and a gray edged white fungus was selected from week 5. A total of 41 microbial isolates were obtained from this enrichment.

Turbidity also appeared in flasks of enrichment medium that were inoculated with soil sample CB10. The medium took on a greenish color within one and one-half weeks, turned brownish-green after two weeks, and then stayed that color throughout the rest of the incubation period. The oily film that remained on the surface of the medium during enrichments inoculated with the other soils disappeared during this enrichment. However, the creosote aroma decreased only slightly after five weeks of incubation. The colonies of bacteria that were obtained when aliquots of the CB10 enrichments were plated during the first three weeks of the enrichment were yellow. Most of the colonies were cream-white at four weeks, whereas they were cream or colorless at five weeks. The colonies at five weeks also tended to spread over the surface of the agar. A total of 37 microbial isolates were obtained from the CB10 enrichments.

Obvious turbidity did not develop in flasks of enrichment medium inoculated with samples CB11, CB12, or CB13. No changes in the color of the medium were seen during the five-week incubation period, and there was no noticeable decrease in creosote aroma.

All the plate counts were below the detection limit and, as a result, no isolates were obtained from these enrichments.

Turbidity did develop in flasks of enrichment medium that were inoculated with soil sample C. The only other noticeable change during the five-week incubation period was a slight decrease in creosote aroma. The colonies of bacteria obtained when aliquots of the sample C enrichments were plated were cream, yellow, or white in color and did not appear to shift during the experiment. A total of 36 microbial isolates were obtained from the soil sample C enrichment.

Twenty of the isolates from enrichment culture experiments were selected for use in the treatability study (treatment number 3; see below). The cell and colony morphological characteristics of these isolates are shown in Table 3-1 and Table 3-2, respectively. Their physiological characteristics, as determined with the API Rapid NFT system (see Materials and Methods), are listed in Table 3-3.

Sixteen of the isolates selected for use in the treatability study were Gram-negative rod-shaped bacteria; the other four isolates were Gram-positive cocci (Table 3-1). None of the Gram-negative rods were able to ferment glucose, but 11 of them could use glucose aerobically as their sole source of carbon (Table 3-3). All of the Gram-negative rods were able to aerobically utilize at least one of the other compounds offered as sole sources of carbon, and 11 of them could use five or more compounds as carbon sources. Twelve of the Gram-negative rods were oxidase positive. All of these characteristics are typical of those found among a group of Gram-negative bacteria that are called the pseudomonads. Only three of the isolates were tentatively identified by the API testing system: TH135 was identified as *Alcaligenes faecalis*, TH137 as *Achromobacter xylosoxidans*, and TH145 as *Pseudomonas fluorescens*.

Table 3-1. Source and cell morphological traits of isolates selected for treatability study.

Isolate number	Source (soil used in enrichment)	Cell shape	Reaction to Gram stain
TH122	CB02	Coccus	Positive
TH123	CB02	Rod	Negative
TH125	CB02	Rod	Negative
TH128	CB02	Rod	Negative
TH134	C	Coccus	Positive
TH135	C	Rod	Negative
TH137	C	Rod	Negative
TH138	C	Rod	Negative
TH139	C	Rod	Negative
TH140	C	Coccus	Positive
TH141	C	Coccus	Positive
TH145	CB02	Rod	Negative
TH146	CB02	Rod	Negative
TH148	CB02	Rod	Negative
TH149	CB02	Rod	Negative
TH164	CB10	Rod	Negative
TH165	CB10	Rod	Negative
TH198	CB10	Rod	Negative
TH203	CB10	Rod	Negative
TH204	CB10	Rod	Negative

Table 3-2. Colony morphological characteristics of isolates selected for treatability study.

Isolate number	Color	Opacity	Surface	Texture	Edge type	Elevation	Other characteristics
TH122	Light red	Opaque	Glossy	None	Smooth	Flat	None
TH123	Cream	Transparent	Semi-glossy	Blotchy	Very irregular	Flat	Spreader
TH125	Yellow	Transparent	Glossy	Rings	Smooth	Flat	None
TH128	Cream-yellow	Opaque	Glossy	Rings	Irregular	Flat	None
TH134	Cream-yellow	Translucent	Glossy	Rings	Sl. irregular	Flat	None
TH135	Cream	Translucent	Glossy	Rings, rays	Sl. irregular	Flat	None
TH137	Yellow	Transparent	Glossy	Rings	Irregular	Flat	None
TH138	Cream	Translucent	Glossy	None	Feathery	Flat	Spreader
TH139	Light yellow	Transparent	Glossy	Rays	Feathery	Flat	None
TH140	Cream	Translucent	Glossy	Rings	Irregular	Flat	None
TH141	Cream-yellow	Translucent	Glossy	Rings, rays	Smooth	Flat	None
TH145	Cream	Translucent	Glossy	Rings	Smooth	Flat	None
TH146	Cream	Translucent	Glossy	Rays	Irregular	Flat	None
TH148	Cream	Transparent	Glossy	Rings, rays	Sl. irregular	Flat	None
TH149	Cream	Transparent	Glossy	Rings	Smooth	Flat	None
TH164	Yellow	Transparent	Glossy	Blotchy, rings	Irregular	Flat	None
TH165	Yellow	Transparent	Dull	Blotchy, rings	Irregular	Flat	None
TH198	Cream	Opaque	Glossy	Blotchy	Very irregular	Flat	Spreader
TH203	Yellow	Transparent	Semi-glossy	Blotchy, rings	Very irregular	Flat	Spreader
TH204	Cream	Opaque	Glossy	None	Smooth	Flat	None

Table 3-3. Physiological characteristics of isolates selected for treatability study.

Isolate no.	Reactions to API Rapid NFT tests[a]																					NFT no.[b]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
TH122	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	+	-	+	-	0-046-542
TH123	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	-	-	+	-	+	-	1-047-542
TH125	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	0-006-104
TH128	+	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	-	+	-	+	+	1-046-556
TH134	+	-	-	-	-	+	+	+	+	-	+	+	+	+	-	-	-	+	-	-	+	1-476-344
TH135	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	0-000-457
TH137	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+	+	0-040-477
TH138	+	-	-	-	-	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	1-550-777
TH139	+	-	-	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+	-	1-140-473
TH140	+	-	-	-	-	-	-	-	+	+	+	-	+	-	+	+	+	+	+	+	+	1-043-577
TH141	+	-	-	-	-	-	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+	1-042-677
TH145	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	-	+	+	+	+	0-046-557
TH146	+	-	-	-	-	-	-	-	+	+	+	+	+	-	+	-	-	+	-	+	+	1-047-546
TH148	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	+	+	-	+	+	0-046-566
TH149	+	-	-	-	-	-	-	+	+	+	+	+	+	-	+	-	+	+	-	+	+	1-067-566
TH164	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	0-400-470
TH165	+	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	1-400-474
TH198	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	0-000-404
TH203	-	-	-	-	-	-	+	-	+	+	-	-	-	+	+	+	-	-	-	-	-	0-051-610
TH204	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	0-441-004

[a]Specific enzymatic capabilities: 1 = nitrate reductase, 2 = tryptophanase, 3 = glucose fermentation, 4 = arginine dihydrolase, 5 = urease, 6 = esculin hydrolysis, 7 = gelatinase, 8 = β-galactosidase, 21 = oxidase. Aerobic utilization of sole carbon sources: 9 = glucose, 10 = arabinose, 11 = mannose, 12 = mannitol, 13 = N-acetyl-D-glucosamine, 14 = maltose, 15 = D-gluconate, 16 = caprate, 17 = adipate, 18 = L-malate, 19 = citrate, 20 = phenyl-alanine.

[b]NFT no. = 7-digit numerical summary of the 21 test results.

Treatability Study: Microbiological Results

The numbers of culturable microorganisms in each of the five treatment soils were monitored throughout the 23-week treatability study by spread plating on 5% TSA agar (see Materials and Methods). The resulting plate counts are provided in Appendix D, and the data are summarized graphically in Figures 3-1 through 3-5.

The numbers of platable microorganisms in treatments 1, 2, 4, and 5 increased by two to three orders of magnitude (rising to between 10^7 and 10^8 CFU/g wet wt.) during the first two weeks of the treatment period and then either remained constant or decreased very slightly throughout the rest of the study (Figs. 3-1, 3-2, 3-4, and 3-5). The initial

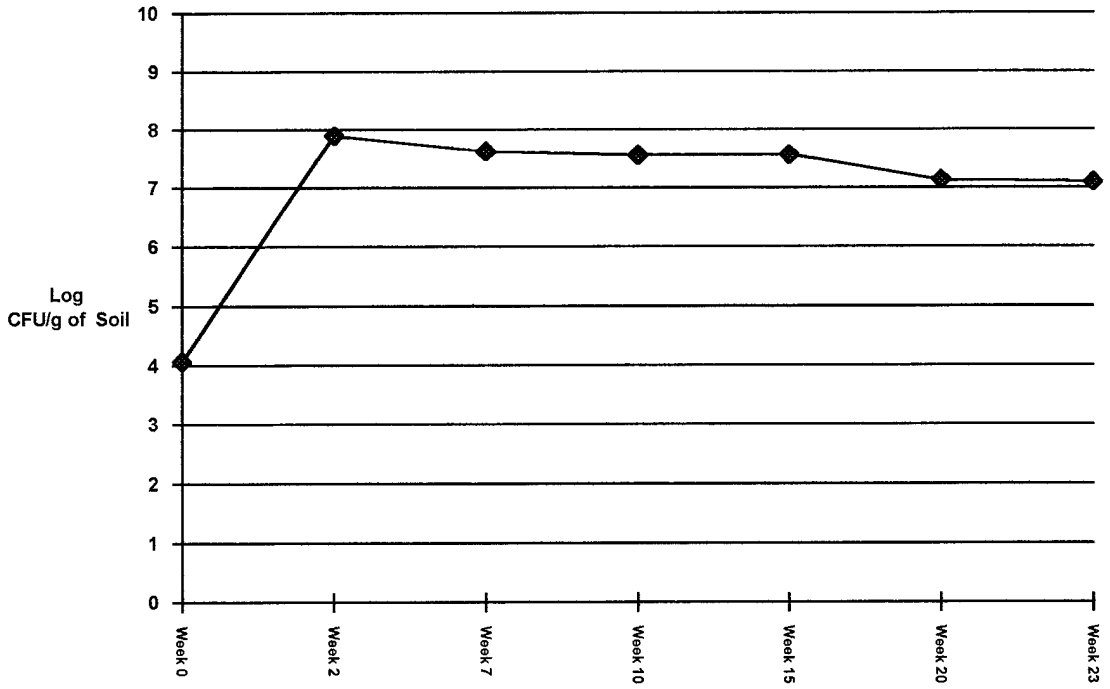

Figure 3-1. Numbers of culturable microorganisms in treatment 1 soil over the 23 week treatability study.

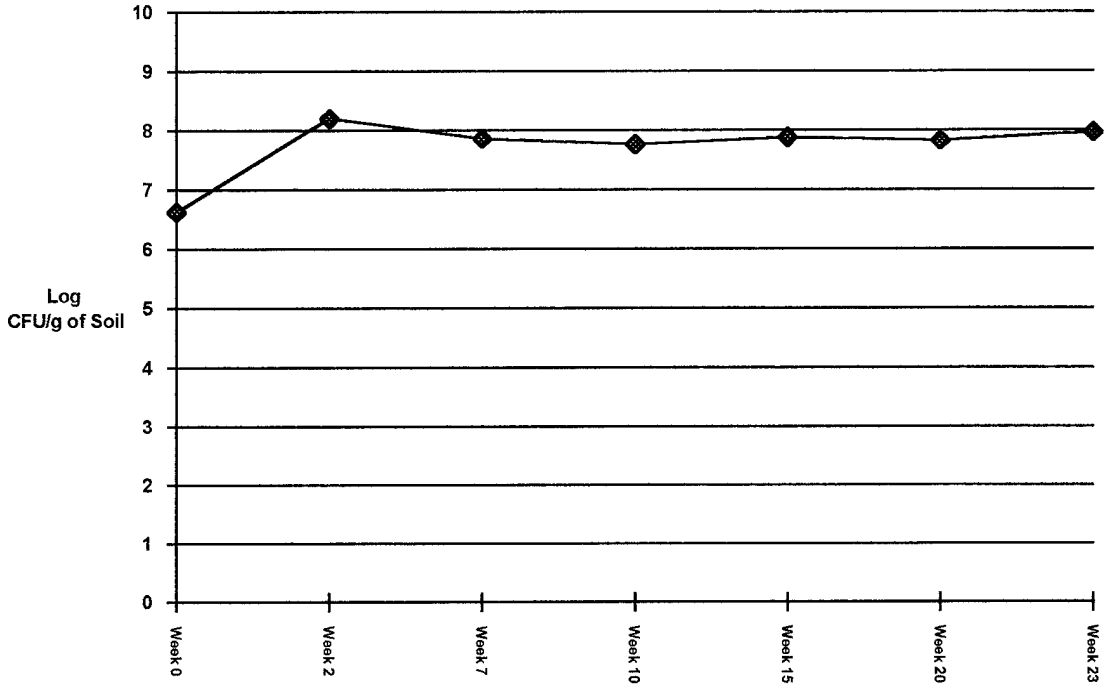

Figure 3-2. Numbers of culturable microorganisms in treatment 2 soil over the 23 week treatability study.

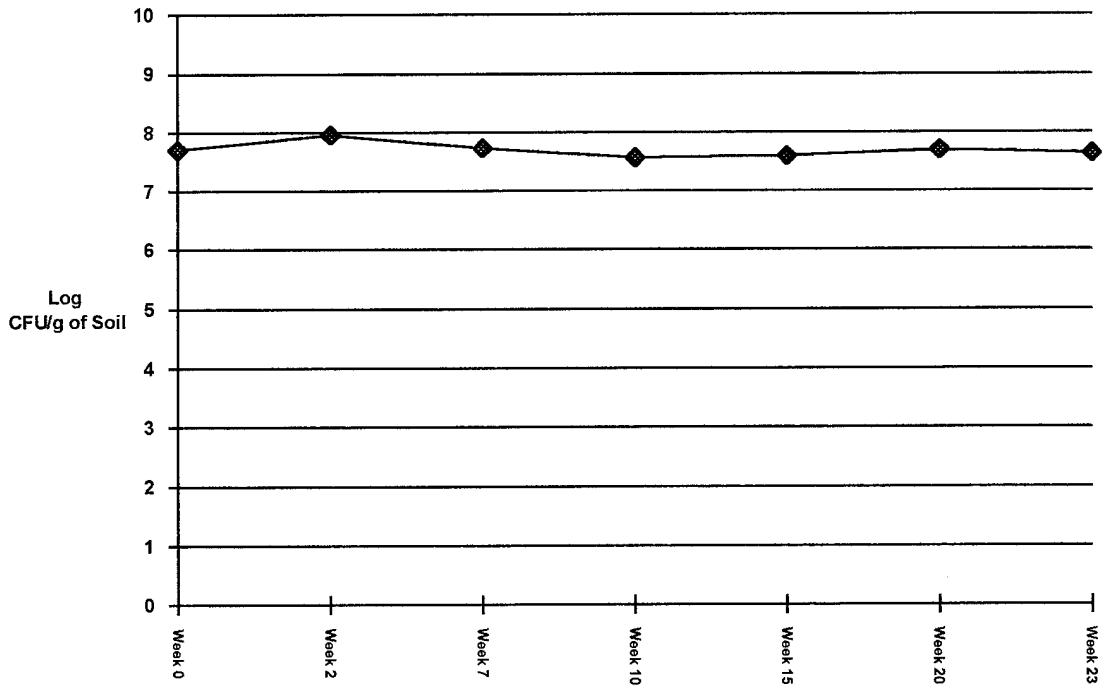

Figure 3-3. Numbers of culturable microorganisms in treatment 3 soil over the 23 week treatability study.

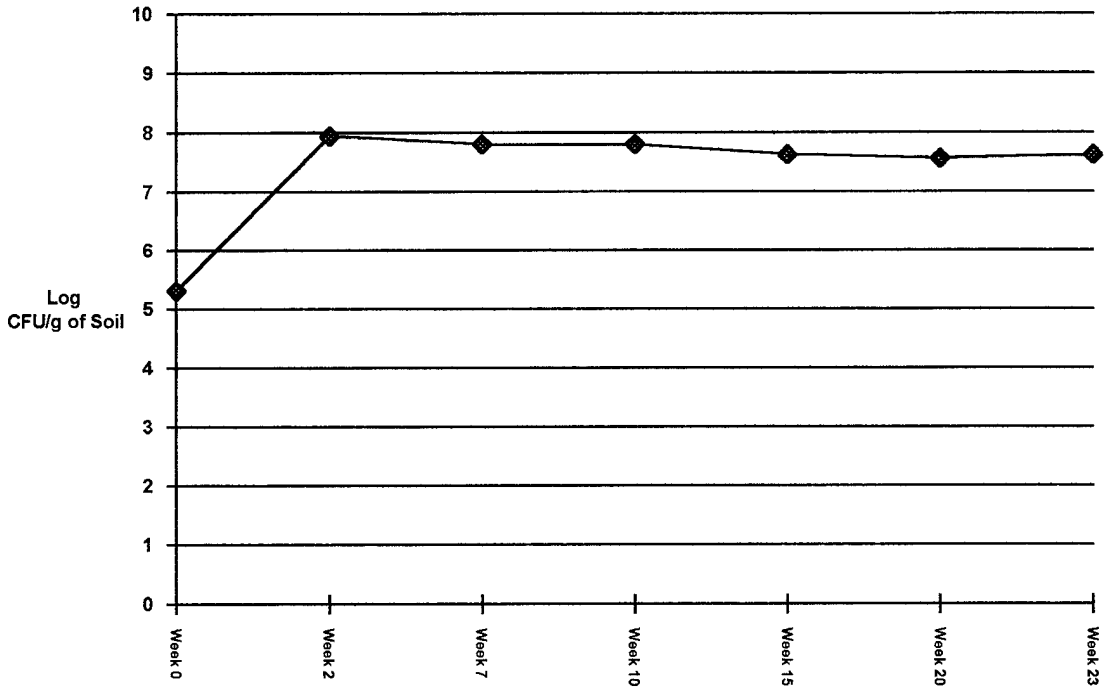

Figure 3-4. Numbers of culturable microorganisms in treatment 4 soil over the 23 week treatability study.

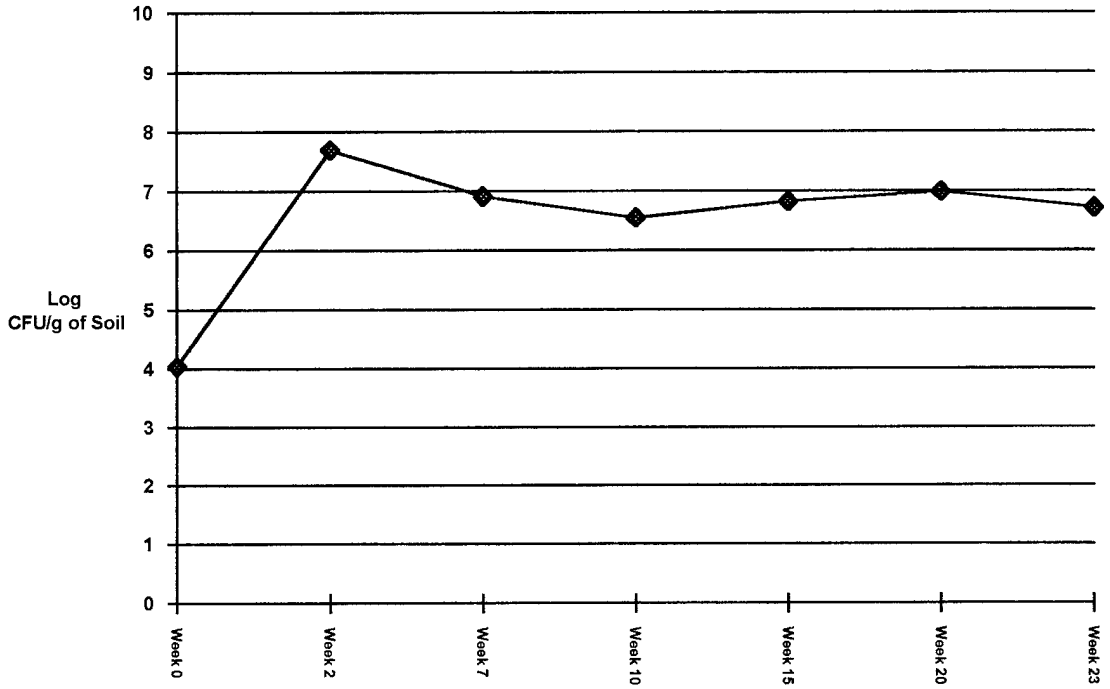

Figure 3-5. Numbers of culturable microorganisms in treatment 5 soil over the 23 week treatability study.

viable count for treatment 3 was comparatively high (> 10^7 CFU/g wet wt.) because this treatment involved the addition of a large number of cultured bacteria isolated from the enrichment experiments described above (see Materials and Methods). Viable counts did not increase in this sample during the first two weeks of the treatment period (Fig. 3-3); rather, they remained at the initial inoculum level throughout the study.

In treatment 2, the white rot fungus (which was deliberately added to the soil in this treatment; see Materials and Methods) was not seen on plates after the initial (week 0) plate count. An attempt was made to isolate the fungus after 15 weeks of incubation by plating the treatment 3 soil on Sabouraud Dextrose Agar (Appendix A), which is selective for fungi. No fungal colonies developed on these plates, thus indicating that the number of fungal propagules in the soil had dropped below the detection limit of the plating procedure (~100 CFU/g wet wt). Apparently, the white rot fungus died off shortly after it was added to the soil at the beginning of the study.

Thirty-four microbial strains (all bacteria) were isolated from the plates used to determine viable numbers of microorganisms in the five treatment soils (above). The cell and colony morphological characteristics of these isolates were recorded, after which the physiological characteristics of 19 selected isolates were determined with the API Rapid NFT system. The other 15 isolates were not analyzed with the API system because: (i) they were isolated early in the 23-week treatment period and, therefore, were unlikely to be creosote degraders, or (ii) they were morphologically identical to isolates that were analyzed. The cell and colony morphological traits of the 19 fully analyzed isolates are shown in Tables 3-4 and 3-5; their physiological characteristics are given in Table 3-6.

Four of the bacterial isolates from the treatment soils (TH224, TH225, TH235, and TH242) appeared to be members of the genus *Streptomyces*, based on their cell and colony morphological traits (Tables 3-4 and 3-5). One of the remaining isolates was a Gram-positive coccus, while the rest (14 strains) were Gram-negative rods. None of the

Gram-negative rods fermented glucose, but eight of them could utilize glucose aerobically as their sole source of carbon (Table 3-6). Four of the Gram-negative rods did not utilize any of the 12 compounds offered as sole carbon sources. The remaining Gram-negative rods used five or more of these compounds aerobically and, thus, possessed physiological traits that are typical of pseudomonads. Five of the Gram-negative rods were tentatively

Table 3-4. Source and cell morphological traits of isolates from treatment soils.

Isolate number	Source (Treatment soil)	Cell shape	Reaction to Gram stain
TH211	Treatment 1	Rod	Negative
TH224	Treatment 1	Filament	Positive
TH225	Treatment 1	Filament	Positive
TH226	Treatment 1	Rod	Negative
TH229	Treatment 1	Coccus	Positive
TH230	Treatment 1	Rod	Negative
TH231	Treatment 1	Rod	Negative
TH232	Treatment 2	Rod	Negative
TH233	Treatment 2	Rod	Negative
TH235	Treatment 3	Filament	Positive
TH236	Treatment 3	Rod	Negative
TH237	Treatment 3	Rod	Negative
TH238	Treatment 3	Rod	Negative
TH239	Treatment 3	Rod	Negative
TH240	Treatment 3	Rod	Negative
TH241	Treatment 4	Rod	Negative
TH242	Treatment 4	Filament	Positive
TH243	Treatment 5	Rod	Negative
TH244	Treatment 5	Rod	Negative

Table 3-5. Colony morphological characteristics of microbial isolates from treatment soils.

Isolate number	Color	Opacity	Surface	Texture	Edge type	Elevation	Other characteristics
TH211	White	Translucent	Glossy	Blotchy	Irregular	Flat	None
TH224	White	Opaque	Dull	Bumpy	Irregular	Slight	Streptomycete
TH225	Cream	Translucent	Glossy	None	Smooth	Slight	Streptomycete
TH226	Yellow	Opaque	Glossy	None	Smooth	Flat	None
TH229	Cream	Transparent	Glossy	None	Smooth	Flat	None
TH230	White	Transparent	Glossy	None	Smooth	Flat	None
TH231	Yellow	Transparent	Semi-glossy	Rays, bumpy	Lobate	Flat	None
TH232	Yellow	Opaque	Glossy	None	Smooth	Slight	None
TH233	Cream	Translucent	Glossy	None	Smooth	Flat	None
TH235	Cream	Opaque	Glossy	None	Smooth	Slight	Streptomycete
TH236	Cream	Opaque	Glossy	None	Smooth	Slight	None
TH237	Yellow	Translucent	Glossy	None	Smooth	Slight	None
TH238	Cream	Translucent	Glossy	None	Smooth	Slight	None
TH239	Cream-white	Opaque	Glossy	None	Smooth	Slight	None
TH240	Cream	Translucent	Semi-glossy	Rays	Feathery	Flat	None
TH241	Cream	Opaque	Glossy	Rings, blotchy	Smooth	Flat	None
TH242	Cream	Opaque	Glossy	None	Smooth	Slight	Streptomycete
TH243	Yellow	Translucent	Glossy	Rings, rays	Smooth	Flat	None
TH244	Light yellow	Transparent	Semi-glossy	Rays	Sl. irregular	Flat	None

Table 3-6. Physiological characteristics of microbial isolates from treatment soils.

Isolate no.	Reactions to API Rapid NFT tests[a]																					NFT no.[b]
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
TH211	+	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	0-046-542
TH224	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	1-047-542
TH225	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0-006-104
TH226	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	1-046-556
TH229	+	-	-	-	-	-	-	-	+	-	+	+	+	-	+	-	-	+	+	+	-	1-476-344
TH230	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	0-000-457
TH231	+	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	0-040-477
TH232	-	-	-	-	-	+	-	+	+	+	-	-	-	+	-	-	-	-	+	-	+	1-550-777
TH233	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	1-140-473
TH235	-	-	-	-	-	+	-	+	+	-	+	-	-	+	-	+	-	-	-	+	+	1-043-577
TH236	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1-042-677
TH237	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	0-046-557
TH238	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1-047-546
TH239	-	-	-	-	-	+	+	+	+	-	+	-	-	+	+	-	-	-	-	-	+	0-046-566
TH240	+	-	-	-	-	+	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	1-067-566
TH241	+	-	-	-	-	-	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	0-400-470
TH242	-	-	-	-	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	+	1-400-474
TH243	+	-	-	+	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+	-	+	0-000-404
TH244	+	-	-	+	-	-	-	-	+	-	-	+	+	-	+	+	-	+	+	-	+	0-441-004

[a]Specific enzymatic capabilities: 1 = nitrate reductase, 2 = tryptophanase, 3 = glucose fermentation, 4 = arginine dihydrolase, 5 = urease, 6 = esculin hydrolysis, 7 = gelatinase, 8 = β-galactosidase, 21 = oxidase. Aerobic utilization of sole carbon sources: 9 = glucose, 10 = arabinose, 11 = mannose, 12 = mannitol, 13 = N-acetyl-D-glucosamine, 14 = maltose, 15 = D-gluconate, 16 = caprate, 17 = adipate, 18 = L-malate, 19 = citrate, 20 = phenyl-alanine.

[b]NFT no. = 7-digit numerical summary of the 21 test results.

identified by the API NFT system: TH244 was identified as *Pseudomonas aeruginosa*; TH232, TH239, and TH240 were identified as *Pseudomonas paucimobilis*; and TH230 was identified as *Pseudomonas vesicularis*. The API system also indicated that isolates TH236 and TH237 might be *Pseudomonas diminuta*, but this identification was given a very low certainty.

Most of the bacterial isolates from the treatment 3 soil produced API Rapid NFT test response patterns that were quite distinct from any of those yielded by the 20 strains that were inoculated into the soil at the beginning of the treatability study. Two isolates (TH236 and TH237) did produce a test pattern (Table 3-6) that was very similar to that produced by one of the inoculum strains (TH198; Table 3-3). However, neither of these isolates possessed the inoculum strain's colony morphological traits (compare Tables 3-5 and 3-4).

Treatability Study: Results of Chemical Analyses

The concentrations of PCP and 17 chemical constituents of creosote in the five treatment soils were determined by GC-MS analysis according to EPA analytical method 8270 (see Materials and Methods). Analyses were performed at 0, 10, and 23 weeks, and the resulting values are detailed in Appendix C. Selected results are presented graphically in Figures 3-6 through 3-17. Calculated degradation rates are listed in Table 3-7.

Six of the analyzed creosote components (naphthalene, acenaphthylene, phenol, 2,4-dimethylphenol, indeno(1,2,3-cd)pyrene, and benzo(g,h,i)perylene) were below the detection limit of the analytical procedure at the start of the treatability study. Two of these compounds (indeno(1,2,3-cd)pyrene and benzo(g,h,i)perylene) were slightly above the detection limit at the end of the study (23 weeks). Thus, their concentrations might have actually increased slightly during the study. The other compounds remained below detect throughout the study.

The concentrations of acenaphthene, fluorene, and anthracene were only slightly above the detection limit at the beginning of the treatability study (Figures 3-6, 3-7, and 3-8, respectively). The concentrations of these compounds dropped below the detection limit in all five treatments within 10 weeks and remained below detect at 23 weeks.

The concentrations of several compounds that initially were well above detection limits decreased substantially during the 23-week treatability experiment. Phenanthrene was initially present at 40,000 µg/kg, but dropped below detect (> 91% reduction) within 10 weeks in all five treatments (Fig. 3-9). The concentration of fluoranthene, initially 93,000 µg/kg, was reduced 91-95% in treatments 1 through 4 after 23 weeks (Fig. 3-10), but only dropped 71% (to 27,000 µg/kg) in treatment 5. Chrysene concentrations were reduced 72% in treatments 3 and 4 after 23 weeks (Fig 3-11); lesser reductions took place in the other treatments. The concentration of benzo(b)fluoranthene dropped only slightly in treatment 1 (12%), but was reduced by 55 to >77% (*i.e.*, below detect) in the other four treatments (Fig. 3-12). Pentachlorophenol was initially present at a comparatively high concentration of 300,000 µg/kg (Fig. 3-13). The concentrations of this compound were reduced substantially (79-89%) in all five treatments. Most of this reduction took place between 0 and 10 weeks, except in treatment 5 (where most of it occurred between 10 and 23 weeks).

The concentrations of pyrene (Fig. 3-14) and benzo(b)fluoranthene (Fig. 3-15) did not decrease appreciably within 23 weeks, with the possible exception of treatment 4 (in which relatively small decreases were seen). The concentrations of benzo(k)fluoranthene (Fig. 3-16) and benzo(a)pyrene (Fig. 3-17) decreased only by small (possibly insignificant) amounts within 23 weeks.

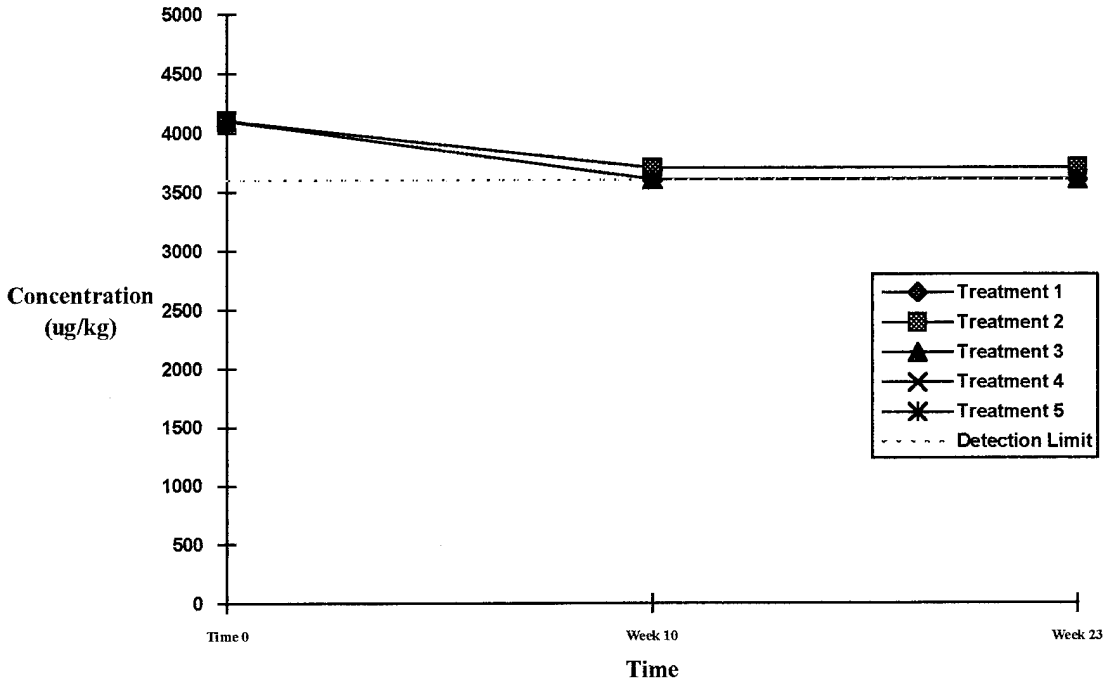

Figure 3-6. Concentrations of acenapthene in treatment soils.

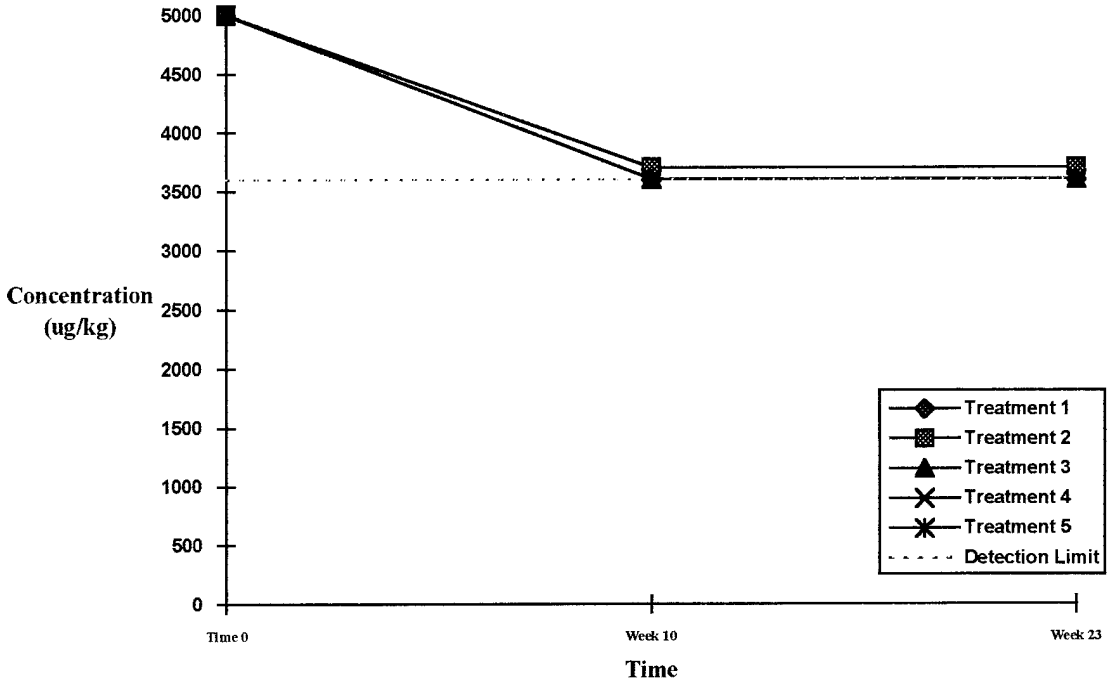

Figure 3-7. Concentrations of fluorene in treatment soils.

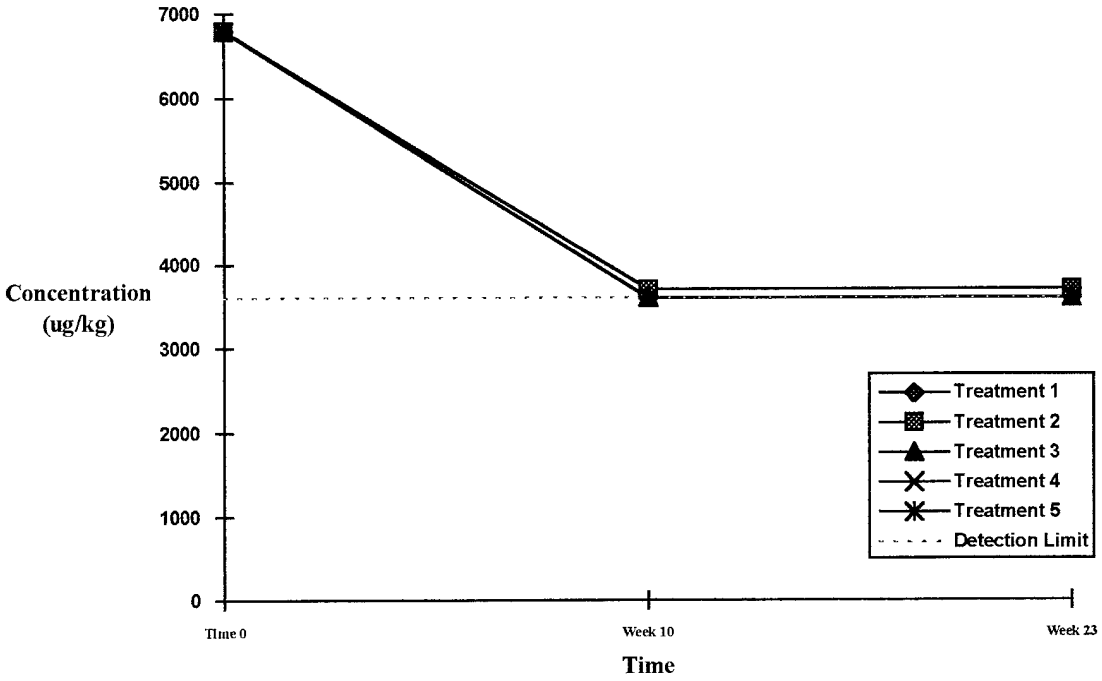

Figure 3-8. Concentrations of anthracene in treatment soils.

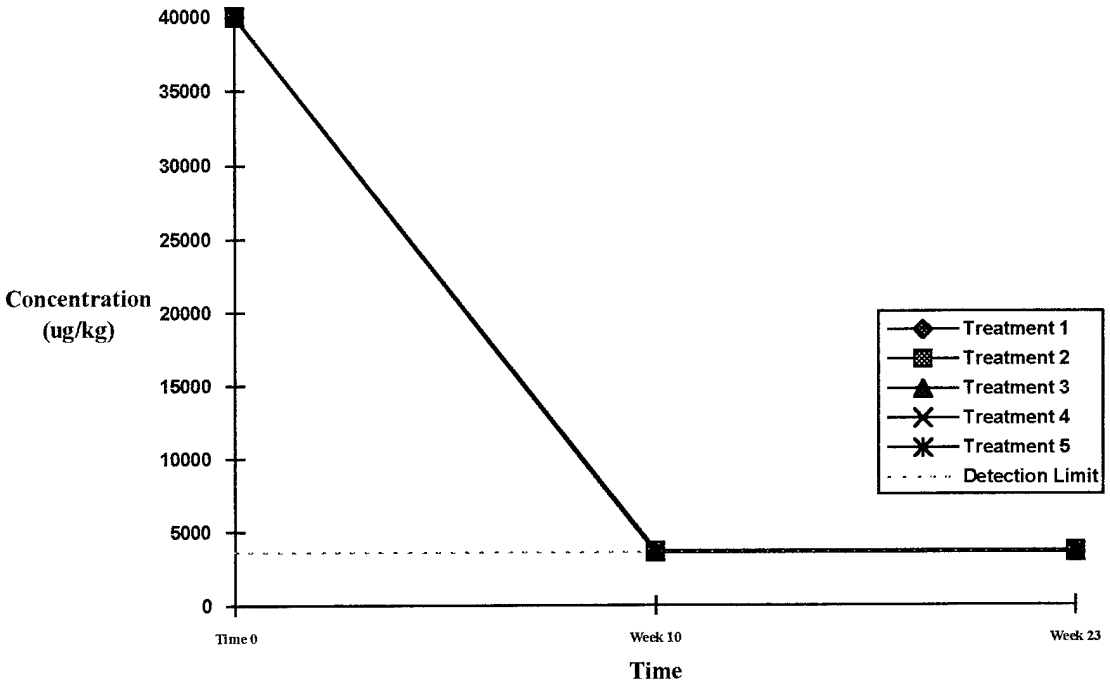

Figure 3-9. Concentrations of phenanthrene in treatment soils.

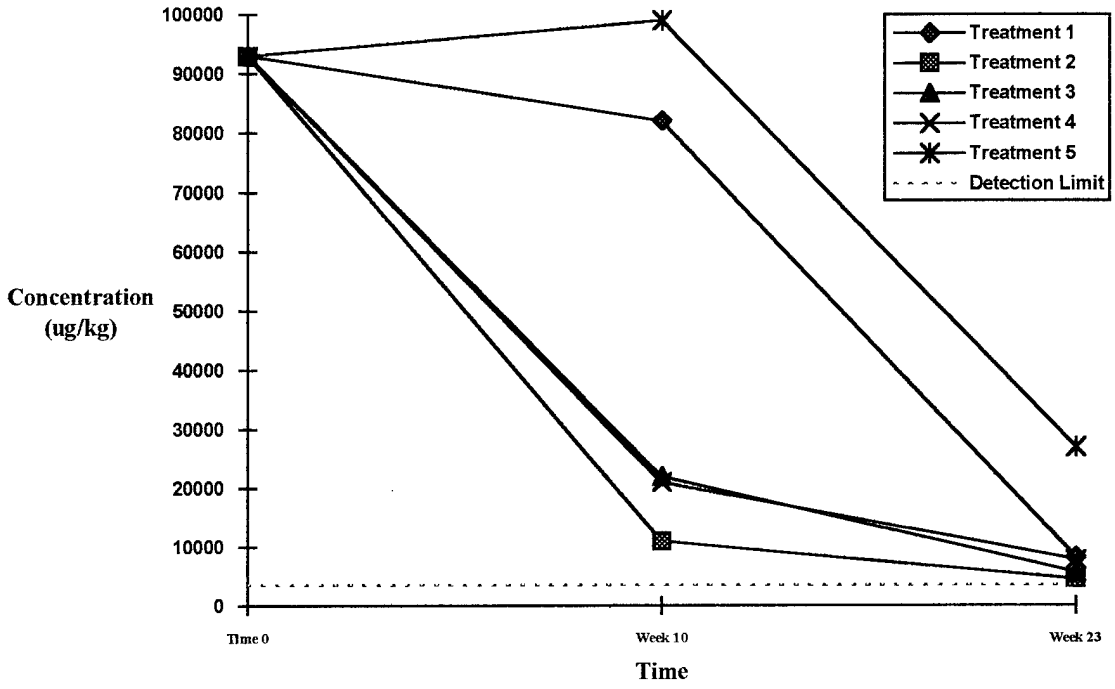

Figure 3-10. Concentrations of fluoranthene in treatment soils.

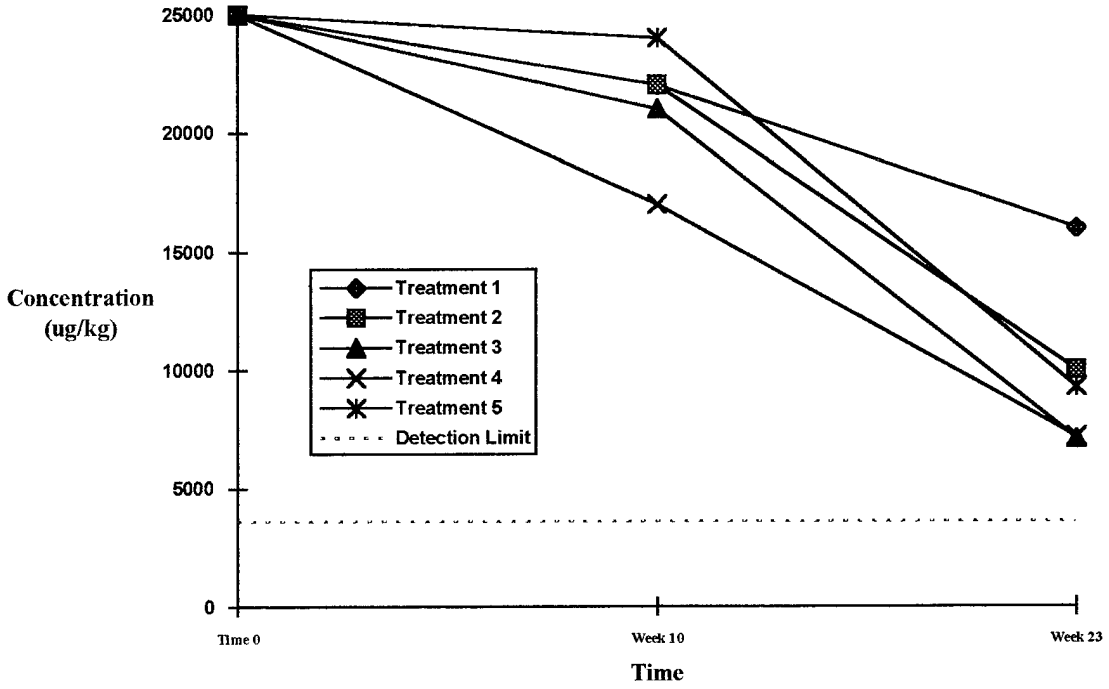

Figure 3-11. Concentrations of chrysene in treatment soils.

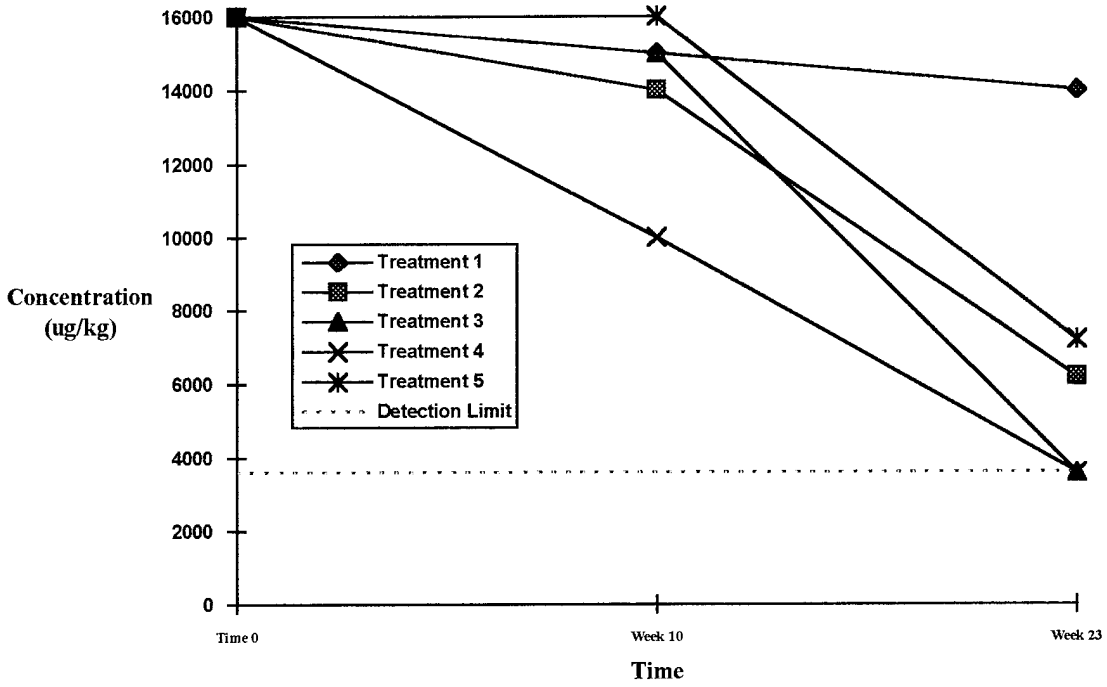

Figure 3-12. Concentrations of benzo(a)anthracene in treatment soils.

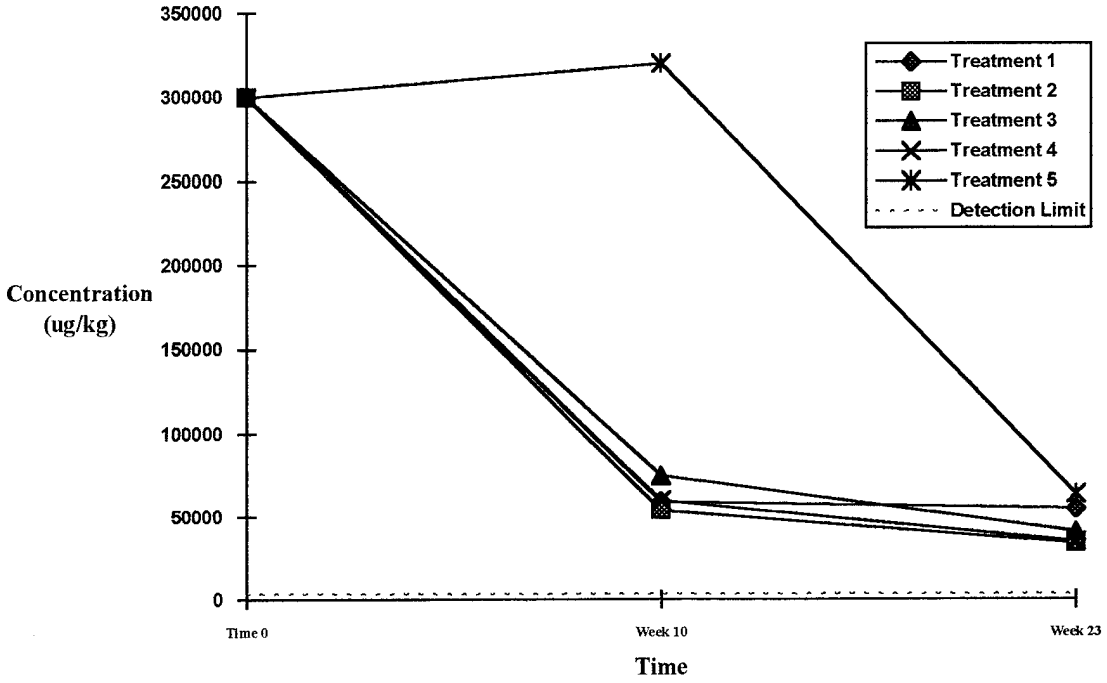

Figure 3-13. Concentrations of pentachlorophenol in treatment soils.

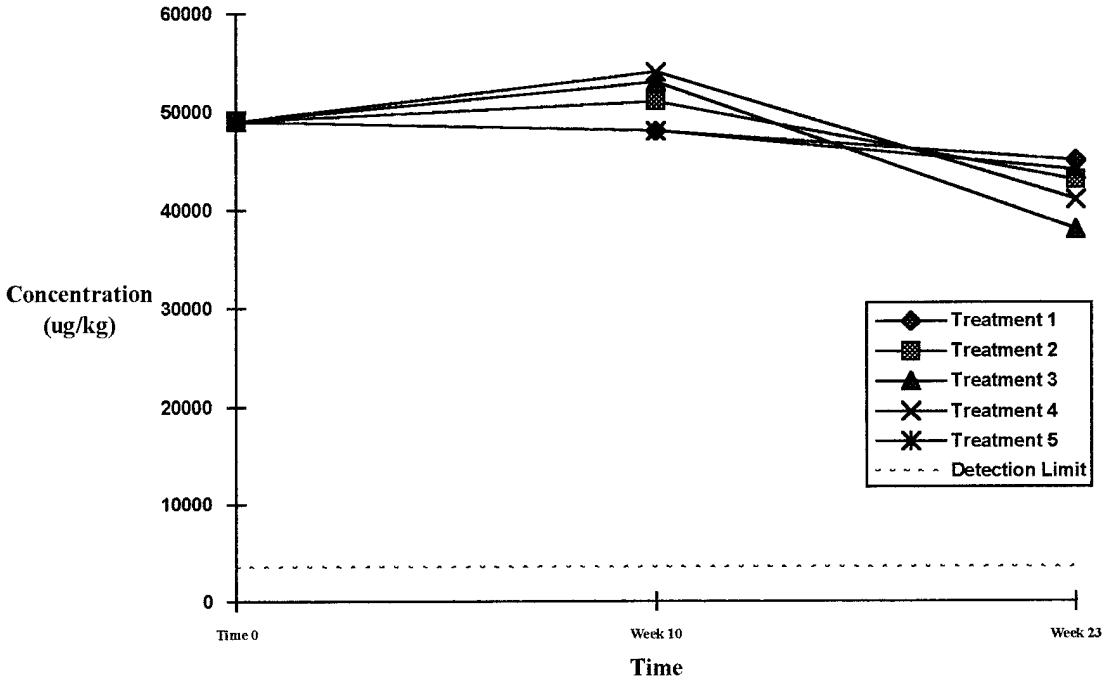

Figure 3-14. Concentrations of pyrene in treatment soils.

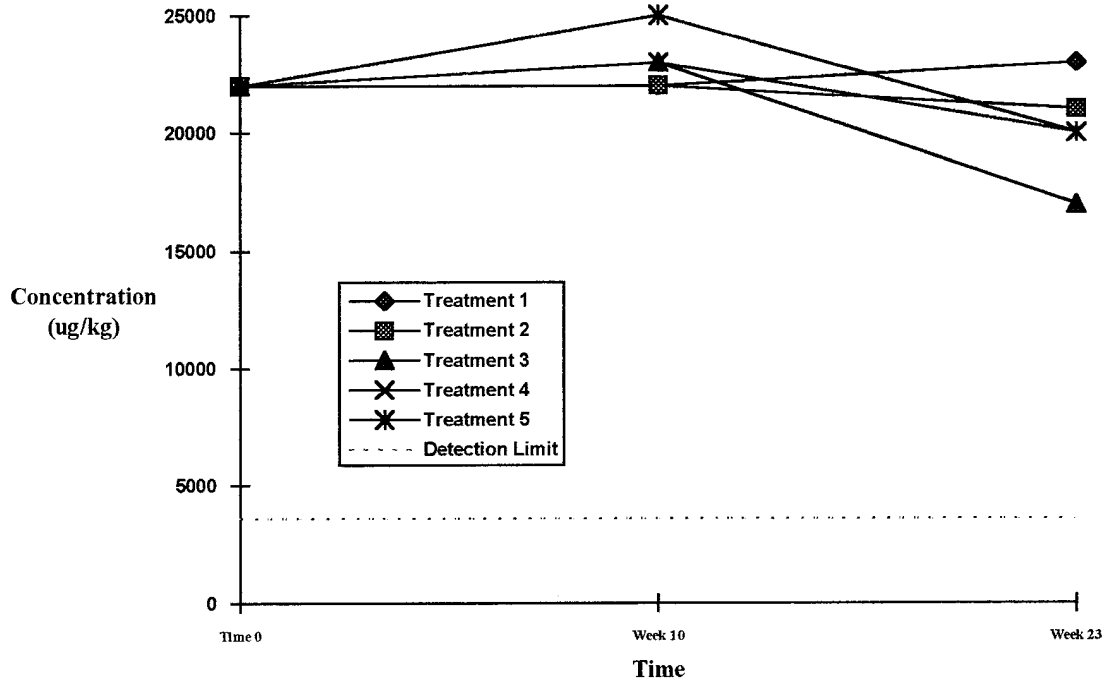

Figure 3-15. Concentrations of benzo(b)fluoranthene in treatment soils.

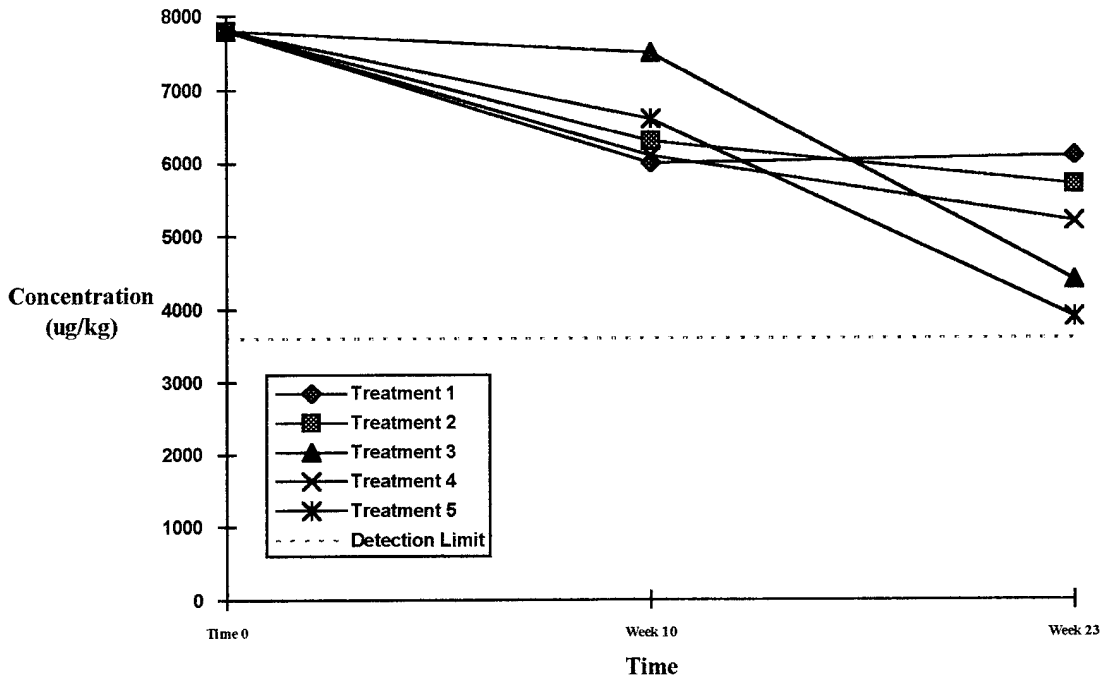

Figure 3-16. Concentrations of benzo(k)fluoranthene in treatment soils.

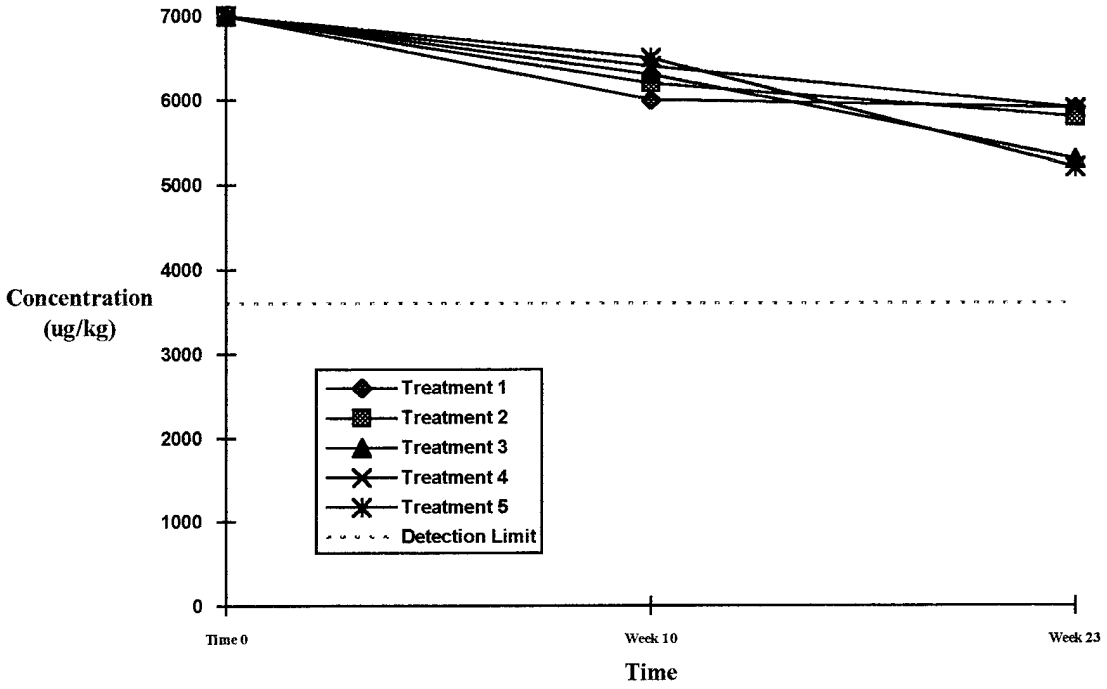

Figure 3-17. Concentrations of benzo(a)pyrene in treatment soils.

Theoretical degradation rates were calculated (in µg/kg of compound removed per week) for all of the chemical compounds whose concentrations decreased during the 23-week treatability study (Table 3-7). Treatment 3 often produced the highest degradation rates, especially with pyrene, chrysene, and benzo(b)fluoranthene. However, differences between treatments were small in many cases. To better assess the difference between each treatment an analysis of variance was performed using the randomized block / repeated measures method. Calculations were accomplished using A Statistical Package for Business, Economics, and the Social Sciences (Dellen/MacMillan, 1992). The analysis of variance table and other statistical information appear in Appendix C. This analysis showed that there was a significant (90% confidence) difference between: Treatment 1 and Treatment 2; Treatment 1 and Treatment 3; Treatment 1 and Treatment 4; Treatment 2 and Treatment 5; Treatment 3 and Treatment 5; and Treatment 4 and Treatment 5. Table 4-8 shows the mean degradation rate for each treatment along with standard deviation, and minimum and maximum values for each treatment without fluoranthene.

Table 3-7. Degradation rates for selected compounds monitored during the treatability study.

Compound	Degradation rate (µg/kg per week) for the following soil treatments				
	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5
Acenapthene	22*	17*	22*	22*	22*
Fluorene	61*	56*	61*	61*	61*
Phenanthrene	1,583*	1578*	1583*	1583*	1583*
Anthracene	139*	135*	139*	139*	139*
Fluoranthene	3,682	3,843	3,796	2,870	261
Pyrene	174	261	478	348	217
Chrysene	391	652	778	774	683
Benzo(a)anthracene	87	426	539	539*	383
Benzo(b)fluoranthene	-43	43	217	87	87
Benzo(k)fluoranthene	74	91	148	113	170
Benzo(a)pyrene	48	52	74	48	78
Pentachlorophenol	10,652	11,565	11,261	11,522	10,261

* = concentration dropped below detection limit; actual degradation rate may be higher than indicated.

Table 3-8. Statistical data for the degradation rates observed by treatment.

	Mean	Standard Deviation	Mimimum	Maximum
Treatment 1	882	2528	-61	10652
Treatment 2	979	2724	-65	11565
Treatment 3	1001	2645	-43	11261
Treatment 4	991	2701	-65	11522
Treatment 5	866	2386	-57	10261

Chapter 4

Discussion

Initial Numbers of Culturable Microorganisms in Soil Samples

The initial plate count for soil sample CB02, from the Wilmington, NC soil farming site, indicated that substantial numbers of culturable (and, therefore, metabolically active) microorganisms were present in this soil. This result was important because it was hoped that creosote-degrading microbial forms had persisted in this soil so that it could be used as an "inoculum" for bioremediation of contaminated soils (the rationale behind treatment 4 in this study). In contrast, the initial plate count for soil sample CB11, from the Camilla, GA wood treating facility site, was below the detection limit of the plating method (~100 CFU/g). A count this low would be considered very unusual for any surface soil, even a dry, sandy soil with a relatively low organic carbon content (Alexander, 1977). The low count for soil CB11, then, is most likely an indication that the contaminants at the sample site were so concentrated or so toxic that they had killed off the indigenous soil microbial populations and had prevented subsequent regrowth of those or other microbial forms.

Enrichment Culture Experiments

The intent of the enrichment cultures was to obtain isolates capable of degrading one or more components of creosote (see Materials and Methods). Soil sample CB02, from the Wilmington, NC site, produced the highest number of isolates, thereby lending credence to the supposition that this soil would contain creosote-degrading strains as the result of a natural enrichment that took place during soil farming.

One of the Camilla wood treating site soils, CB10, also produced several isolates during enrichment. This would be expected because the Camilla soil has been exposed to creosote for many years, easily enough time for enrichment of creosote-degrading species to take place. However, the other three Camilla site soils (CB11, CB12, and CB13) did not produce any isolates during enrichment. Given the lack of culturable microorganisms in one of the samples (CB11; the other two were not plated at the beginning of the study), this would seem to be further evidence that the contaminants in some parts of the Camilla site were so concentrated that even creosote-degrading strains have not been able to grow in the soils there.

Soil sample C also produced several isolates. It is rather unlikely that any natural enrichment for creosote-degraders would have occurred in this soil before it was collected for the present study because the soil had never been exposed to creosote. Rather, it may well be possible to find organisms capable of degrading at least some creosote compounds in almost any typical soil (see Alexander, 1977; Atlas and Bartha, 1992). Whether or not these microbes can degrade contaminants as effectively as those that predominate the soil microbial community after many years of exposure to creosote (*i.e.*, that grow up during a natural enrichment) remains to be seen.

Most of the enrichment culture isolates used for the treatability study were Gram-negative, rod-shaped bacteria that possessed an oxidative (as opposed to a fermentative) metabolism and the ability to utilize degrade different organic compounds as sole sources of carbon. These traits are significant because they are characteristic of a loosely defined group of bacteria known informally as the pseudomonads. The pseudomonads, especially those that are members of the genus *Pseudomonas* and closely related genera, have long been associated with the degradation of organics, including complex compounds and toxic compounds that frequently occur as environmental contaminants (Atlas and Bartha, 1992; Silver et al., 1990). Of the major groups of bacteria that are common in surface soils, it is

the pseudomonads that are most likely to be able to degrade creosote components and that would be most likely to grow up in an enrichment medium that contained creosote as the sole source of carbon.

It remains to be seen whether any of the enrichment cultures isolates actually can degrade creosote compounds. To show this conclusively, it would be necessary to grow each isolate in the presence of creosote and monitor the fate of all creosote components by GC-MS or some other type of analysis. Tedious and expensive studies with radiolabeled compounds might then be required to prove that any missing components disappeared as a result of microbial degradation (as opposed to volatilization or some other non-biological process). Because the purpose of this study was merely to determine the value (if any) of adding a microbial inoculum based on enrichment isolates to contaminated soil in order to promote bioremediation, extensive degradation studies on individual isolates were beyond its scope. Nevertheless, further characterization of these isolates would be an interesting and useful follow-up to the present investigation.

The API Rapid NFT system only identified three of the enrichment culture isolates. This is not surprising, considering that the system was actually designed for identification of bacteria isolated from clinical sources rather than from non-clinical environments. (The system's primary use in the case of environmental strains is for relatively rapid acquisition of basic physiological data.) Those identifications that were obtained must be considered tentative because the manufacturer of the Rapid NFT system guarantees accuracy only in the case of clinical isolates. Nevertheless, the three identifications are believable; all three are common soil species, and two of them would be considered pseudomonads.

Treatability Study - Microbiological Results

The numbers of culturable microbes in treatment soils 1, 2, 4, and 5 increased by two to three orders of magnitude during the first two weeks of the treatability study (see

Figures 3-1, 3-2, 3-4, and 3-5). Most of the soil samples that were combined to produce the soil used in the treatability study were compacted (which limits exchange of oxygen) and/or dry, and they had been stored in that condition for some time (see Materials and Methods). One would expect the soil microbial populations to be comparatively sparse and inactive under these conditions. Oxygen was introduced into the soils as they were mixed and prepared for the treatability experiment, but the lack of moisture would have prevented extensive microbial growth during that step of the experiment. The extensive growth seen shortly after the treatability macrocosms were set up, then, was most likely just a response to the added water. This type of response is seen almost any time water is added to a dry soil and is considered a standard phenomenon of soil microbial ecology (Alexander, 1977).

After the initial growth response (above), the numbers of culturable organisms in treatment soils 1, 2, 4, and 5 remained fairly high ($>10^7$ CFU/g) throughout the 23-week treatability study. Therefore, the conditions used during the study (periodic aeration and addition of water) were at least sufficient to support relatively large populations of active microorganisms for an extended period of time. This is important with respect to removal of contaminants because, in any bioremediation procedure, contaminants will be degraded rapidly and extensively only if large populations of actively degrading microorganisms can be maintained at all times.

The number of culturable microorganisms in treatment soil 3 did not increase after the macrocosm for this treatment was set up (Fig. 3-3). In all likelihood, no increase was seen because the microbial inoculum used in this treatment brought the initial total number of culturable cells up to the maximum level ($\sim 10^7$ CFU/g) that could be maintained in the soil under the conditions provided throughout the treatability study. The large inoculum also would have masked any initial growth response that did take place on the part of the native microorganisms in the soil because their initial numbers were very much lower.

The total numbers of culturable microorganisms in treatment 3 remained constant throughout the 23-week treatability study (Fig. 3-3), but there must have been a shift in the types of organisms that were present in the population. None of the microorganisms isolated from this treatment (by plating) after 23 weeks were similar (in morphological or physiological traits) to any of the 20 organisms used in the inoculum (compare Tables 3-5 and 3-6 with Tables 3-2 and 3-3, respectively). These results imply that the organisms in the inoculum failed to persist throughout the 23-week treatability period, eventually being replaced by native soil strains that grew up in their place as they died off. It is important to realize, however, that plating detects only the numerically predominant organisms in a mixed population. Therefore, some or all of the 20 inoculum strains could still have been present at the end of the study, but only in numbers too low to be detected by plating. It is not known for sure when the inoculum organisms decreased in numbers, so they could have played a significant role in the degradation of some contaminants (see below) before they died back.

Unlike the inoculum strains in treatment 3, the white rot fungus used for treatment 2 could not have played any direct role in the degradation of contaminants. This organism apparently died off very shortly after being added to the soil (see Results). At the most, it might have contributed some nutrients (released as the fungal cells died) that were used by the other microorganisms in the soil. It is not known why the white rot fungus died off so quickly. Perhaps it was unable to compete with the bacterial populations already present in the soil. If the soil contained a high concentration of nitrogen, this could have inhibited the fungus as well (Mileski, 1988). Unfortunately, this possibility could not be tested in the present study because all the treatment soil was needed for the GC-MS chemical analyses.

Treatability Study: Chemical Analyses

The GC-MS analyses showed that the concentrations of PCP and some creosote compounds were reduced by as much as 95% over the 23-week treatment period. These decreases were almost certainly the result of microbial degradative activities. It is highly unlikely that volatilization could account for such extensive decreases, considering that the compounds monitored by GC-MS were only semi-volatile at most. The fact that many of the bacteria isolated from the various treatments at the end of the study possessed traits that are characteristic of pseudomonads also suggests that microbial degradation occurred (see above). Moreover, some of isolates were tentatively identified by the API system as *Pseudomonas aeruginosa* or *Pseudomonas paucimobilis*, two species that have been reported to degrade a variety of aromatic and phenolic compounds, including some that are commonly present in creosote (Silver et al., 1990).

The GC-MS analyses also showed that the concentrations of some of the creosote compounds that were monitored either remained constant or decreased just slightly during the 23-week treatment period. For the most part, this was the case in all five treatments. The slight decreases may or may not have been significant, but that would not make any difference from a practical standpoint. It is very unlikely that prolonged treatment by any of the bioremediation methods tested here could bring the concentrations of the relatively recalcitrant creosote constituents below the detection limit in a period of time that would be acceptable to a regulatory agency.

The effectiveness of inoculation with the white rot fungus (treatment 2) could not be evaluated in this study because the fungus died so quickly after it was added to the soil (see above). Additional research would be required to identify treatment conditions that would better promote the growth and persistence of the fungus.

Several creosote components were degraded more rapidly in treatment 3 than in the other four treatments (Table 3-7). These results indicate that addition of the cultured

inoculum (consisting of isolates from enrichment cultures) probably stimulated removal of certain contaminants, at least during the initial weeks of the study. (As noted above, the inoculum organisms were eventually replaced by native species and, therefore, could not have had much effect on degradation during the latter part of the study.) However, the differences between treatment 3 and the other treatments were slight. Given the cost of preparing an inoculum large enough to treat contaminated soil on a typical field scale (*e.g.*, 10,000-500,000 cubic yards), the small gains that might be realized by using the inoculum probably would not justify the extra expense of this approach over simple land farming.

Treatment 4, which used soil from a land farming site as an "inoculum", generally worked as well as treatment 3 (Table-3-7 and Figures 3-6 through 3-17). Depending on the distance over which the land-farmed soil would have to be hauled to a new treatment site, this type of inoculum might be considerably less expensive than a cultured one (as in treatment 3). Therefore, this approach might be worth trying on a larger scale at a site where land-farmed soil is readily available.

With the possible exception of benzo(k)fluoranthene, addition of sodium nitrate (treatment 5) did little or nothing to stimulate biodegradation of creosote components (Table 3-7). In fact, nitrate addition may have greatly inhibited the biodegradation of fluoranthene for some reason (Fig. 3-10). These findings show that, in the case of the Camilla site soil, there is no reason to include nitrate amendments in the bioremediation procedure.

Perhaps the most dramatic reduction of contaminant (in all five treatments) was seen in the case of PCP (Fig. 3-17). Except for treatment 4, however, almost all of this reduction occurred during the first 10 weeks of the study; there was only a slight further reduction during the last 13 weeks. These results imply that prolonged treatment would be unlikely to reduce the levels of PCP to acceptable levels in a reasonable period of time.

Nevertheless, the rapid initial reduction might be useful in a situation were the soil was then treated further by a different method.

Other researchers have achieved varying levels of success in this area of research as well. A hyperfiltration unit can remove up to 100% of many of the compounds in creosote from contaminated water. PCP removal was demonstrated to be better than 97% (Middaugh, Thomas, et al., 1993). Using bacteria in shake flasks, Mueller et al. (1991b) were able to remove up to 100% of the lower-molecular-weight PAHs from contaminated groundwater in 14 days. They met minimal success against the higher-molecular-weight PAHs (57%), and were unable to remove any PCP from the contaminated water. Using a bioreactor, Middaugh, Lantz, et al. (1993) were able to remove 81% of the PCP from contaminated water within 15 days. They were able to remove 77.3% of the PAHs in creosote, using the same device. Fluoranthene enriched bacteria were able to remove up to 100% of a number of creosote components, for example phenanthrene, benzo(a)pyrene and fluorene (Mueller, 1989a). Mileski (1988) reported up to 97% reduction of low concentrations of PCP by white rot fungus under nitrogen poor conditions in a liquid medium. However, higher concentrations of PCP were degraded to a much lesser extent, probably due to the toxicity of the compound against the fungus.

Conclusions Regarding the Feasibility of Bioremediation

For any of the biormediation methods tested in this study to be practical for actual field applications, two minimal requirements would have to be met: (i) all contaminants of concern to EPA would have to be reduced below the levels currently permitted by EPA and/or other regulatory agencies that might oversee a contaminated site; and (ii) all of the contaminants would have to be reduced to those levels in a period of time that is not only acceptable to the regulatory agencies, but would also keep the total cost of project below that of incineration or other physical remediation methods (see Introduction). None of the

methods tested in this study appear likely to meet these criteria (based on the findings of the 23-week treatability experiments) and, therefore, none of them would be likely to be adapted by environmental engineering companies as the sole method for remediation of a contaminated wood treating site.

Because substantial reductions in the concentrations of PCP and some creosote components were seen, any of the methods tested here might be useful in field situations if they were combined with other approaches. For example, treatment 1 could be followed by treatment in a bioreactor that contained microorganisms specifically designed to attack the remaining (*i.e.*, more recalcitrant) creosote components. Treatment 1 would probably be the most practical approach in any such application because it is the least expensive of the five and only slight (possibly insignificant) improvements were obtained by resorting to the more expensive methods.

Appendix A

Microbiological Culture Media

Table A-1. 5% Peptone-Tryptone-Yeast Extract-Glucose Broth (5% PTYG).

Component	Quantity
Dextrose (D-glucose)	10.0 g
Difco Yeast Extract	10.0 g
Difco Peptone	5.0 g
Difco Tryptone	5.0 g
$MgSO_4.7H_2O$	0.6 g
$CaCl_2.2H_2O$	0.07 g
Distilled water	1.0 liter

Table A-2. Phosphate Buffered Saline Solution (PBS).

Component	Quantity
$Na_2HPO_4 \cdot 7H_2O$	6.67 g
$NaH_2PO_4 \cdot H_2O$	0.67 g
NaCl	25.5 g
Distilled Water	3.0 L

Note: Allow to continue to stir for five minutes after all of the NaCl has dissolved.

Table A-3. Pyrophosphate.

Component	Quantity
$Na_4P_2O_7 \cdot 10\ H_2O$	1.00 g
Distilled H_2O	1.00 L

Note: Adjust pH to 7.0.

Table A-4. Sabouraud Dextrose Agar.

Component	Quantity
Difco Neopeptone	5.00 g
Dextrose	20.00 g
Agar (granulated)	7.50 g
Distilled Water	300 ml

Note: Adjust pH to 5.6 with 5N HCl. Autoclave and hand pour at 45^{o}C.

Table A-5. Stock Salts Solution for Hunter's Mineral Base.

Component	Quantity
EDTA	2.5 g
$ZnSO_4 \cdot 7H_2O$	10.95 g
$FeSO_4 \cdot 7H_2O$	5.0 g
$MnSO_4 \cdot H_2O$	1.54 g
$CuSO_4 \cdot 5H_2O$	0.39 g
$Co(NO_3)_2 \cdot 6H_2O$	0.24 g
$Na_2B_4O_7 \cdot 10H_2O$	0.17 g
Distilled Water	1.0 L

Note: Adjust pH to 7.0 .

Table A-6. Hunter's Mineral Base.

Component	Quantity
Nitrilotriacetic Acid	10.0 g
KOH -- adjust pH to 5.0	7.28 g
$MgSO_4$	14.45 g
$CaCl_2 \cdot 2H_2O$	3.33 g
$(NH_4)_6 \cdot 7H_2O$	0.0093 g
$FeSO_4 \cdot 7H_2O$	0.0990 g
Stock Salts Solution for Hunter's Mineral Base	50 ml
Distilled Water	950 ml

Note: Adjust pH to 6.8 using KOH and H_2SO_4.

Table A-7. Phosphate Solution.

Component	Quantity
KH_2PO_4	68.0 g
NaH_2PO_4	71.0 g
Distilled Water	1.0 L

Note: Adjust pH to 6.8 using NaOH.

Table A-8. Stanier's Standard Mineral Base.

Component	Quantity
Phosphate solution	400 ml
Hunter's Mineral Base	20 ml
$(NH_4)_2SO_4$	1.0 g
Distilled Water	940 ml

Table A-9. 5% Trypticase Soy Broth (TSB).

Component	Quantity
Becton Dickson Trypticase Soy Broth (Powdered)	1.5 g
Distilled Water	1.0 L

Table A-10. 5% Trypticase Soy Agar (TSA).

Component	Quantity
5% TSA Broth	1.0 L
Becton Dickson Agar (Granulated)	15.0 g

Table A-11. White Rot Medium.

Component	Quantity
Pulse ground wood chips.	Approximately 0.5 L (final volume)
Sterile 5% PTYG	50 ml
H_2O	130 ml

Notes: The ground chips should range from a fine powder to chips sized 10 X 3 X 3 mm. Autoclave the chips in a foil covered 2,800 ml Erlenmeyer flask. Add water and 5% PTYG until the wood is visibly moist but not wet.

Appendix B

Detailed Description of Physiological Tests in the API Rapid NFT Test Kit

Table B-1. API Rapid NFT tests for specific enzymatic capabilities.

Biochemical test	Reactive ingredient	Physio-chemical principle
NO_3 Nitrate Reduction	KNO_3 KNO_3 + zinc dust	Reduction of NO_3 to NO_2 Reduction of NO_3 to N_2
Tryptophanase	Tryptophane + James Reagent	Tryptophane metabolism yields indole, which reacts with the James Reagent
Glucose fermentation	Glucose	Glucose fermentation lowers the pH. Brom thymol blue is the indicator.
Arginine dihydrolase	Arginine	Arginine is transformed into ornithine, ammonia and CO_2, raising the pH. Phenol red is the indicator.
Urease	Urea	Urease releases ammonia from urea, raising the pH. Phenol red is the indicator.
Esculin hydrolysis	Esculin	Esculin is hydrolized by beta-glucosidase into glucose and esculetine. This reacts with iron salt to give a black indication.
Gelatinase	Kohn charcoal Gelatin	Proteolytic enzymes liquefy the gelatin, releasing the charcoal. Black indication.
Beta-galactosidase	PNPG + IPTG	Hydrolysis of p-nitro-phenyl-galactopur-anoside by beta-galactosidase releases yellow para-nitrophenol. Isopropythio-galactopyranoside is used as an inducer.

Table B-2. Tests for aerobic assimilation of sole carbon sources.

Assimilation test	Reactive ingredient
D-Glucose	D-Glucose
L-Arabinose	L-Arabinose
D-Mannose	D-Mannose
D-Mannitol	D-Mannitol
N-Acetyl-D-glucosamine	N-Acetyl-D-glucosamine
Maltose	Maltose
D-Gluconate	D-Gluconic acid
Caprate	Capric acid
Adipate	Adipic acid
L-Malate	L-malic acid
Citrate	Citric acid
Phenylacetate	Phenylacetic acid

Appendix C

Gas Chromatography - Mass Spectrophotometry (GC-MS) Analytical Results and Statistical Analysis of GC-MS Results

Table C-1. Concentrations of PCP and 17 creosote components in treatment soil 1.

	Concentration (µg/kg dry wt) at these times		
Compound	Time 0	Week 10	Week 23
Naphthalene	<3600	<3600	<3600
Acenaphthylene	<3600	<3600	<3600
Acenaphthene	4100	<3600	<3600
Fluorene	5000	<3600	<3600
Phenanthrene	40000	<3600	<3600
Anthracene	6800	<3600	<3600
Fluoranthene	93000	82000	8300
Pyrene	48000	48000	45000
Chrysene	25000	22000	16000
Benzo(a)anthracene	16000	15000	14000
Benzo(b)fluoranthene	22000	22000	23000
Benzo(k)fluoranthene	7800	6000	6100
Benzo(a)pyrene	7000	6000	5900
Indeno(1,2,3-cd)pyrene	<3600	<3600	5000
Benzo(g,h,i)perylene	3600	<3600	4900
Phenol	<3600	<3600	<3600
2,4-Dimethylphenol	<3600	<3600	<3600
Pentachlorophenol	300000	59000	55000

Table C-2. Concentrations of PCP and 17 creosote components in treatment soil 2.

	Concentration (µg/kg dry wt) at these times		
Compound	Time 0	Week 10	Week 23
Naphthalene	<3600	<3700	<3700
Acenaphthylene	<3600	<3700	<3700
Acenaphthene	4100	<3700	<3700
Fluorene	5000	<3700	<3700
Phenanthrene	40000	<3700	<3700
Anthracene	6800	<3700	<3700
Fluoranthene	93000	11000	4600
Pyrene	48000	51000	43000
Chrysene	25000	22000	10000
Benzo(a)anthracene	16000	14000	6200
Benzo(b)fluoranthene	22000	22000	21000
Benzo(k)fluoranthene	7800	6300	5700
Benzo(a)pyrene	7000	6100	5800
Indeno(1,2,3-cd)pyrene	<3600	<3700	5200
Benzo(g,h,i)perylene	3600	<3700	5100
Phenol	<3600	<3700	<3700
2,4-Dimethylphenol	<3600	<3700	<3700
Pentachlorophenol	300000	54000	34000

Table C-3. Concentrations of PCP and 17 creosote components in treatment soil 3.

	Concentration (µg/kg dry wt) at these times		
Compound	Time 0	Week 10	Week 23
Naphthalene	<3600	<3600	<3600
Acenaphthylene	<3600	<3600	<3600
Acenaphthene	4100	<3600	<3600
Fluorene	5000	<3600	<3600
Phenanthrene	40000	<3600	<3600
Anthracene	6800	<3600	<3600
Fluoranthene	93000	22000	5700
Pyrene	48000	53000	38000
Chrysene	25000	21000	7100
Benzo(a)anthracene	16000	15000	3600
Benzo(b)fluoranthene	22000	23000	17000
Benzo(k)fluoranthene	7800	7500	4400
Benzo(a)pyrene	7000	6300	5300
Indeno(1,2,3-cd)pyrene	<3600	<3600	4600
Benzo(g,h,i)perylene	3600	<3600	4300
Phenol	<3600	<3600	<3600
2,4-Dimethylphenol	<3600	<3600	<3600
Pentachlorophenol	300000	75000	41000

Table C-4. Concentrations of PCP and 17 creosote components in treatment soil 4.

	Concentration (µg/kg dry wt) at these times		
Compound	Time 0	Week 10	Week 23
Naphthalene	<3600	<3600	<3600
Acenaphthylene	<3600	<3600	<3600
Acenaphthene	4100	<3600	<3600
Fluorene	5000	<3600	<3600
Phenanthrene	40000	<3600	<3600
Anthracene	6800	<3600	<3600
Fluoranthene	93000	21000	7900
Pyrene	48000	54000	41000
Chrysene	25000	17000	7200
Benzo(a)anthracene	16000	10000	<3600
Benzo(b)fluoranthene	22000	23000	20000
Benzo(k)fluoranthene	7800	6100	5200
Benzo(a)pyrene	7000	6400	5900
Indeno(1,2,3-cd)pyrene	<3600	<3600	5100
Benzo(g,h,i)perylene	3600	<3600	4600
Phenol	<3600	<3600	<3600
2,4-Dimethylphenol	<3600	<3600	<3600
Pentachlorophenol	300000	60000	35000

To find if there was a difference between treatments, a rate of degradation was calculated. It is recognized that there is usually a hyperbolic change in the concentration over time for most degradation problems. For the purpose of comparison, though, the change in concentration can be assumed to approach a straight line over a short test period. The rate was calculated by subtracting the concentration of a particular compound from its initial concentration at time zero and dividing the result by 23 weeks and reversing the sign ($\text{Rate} = -\frac{[\text{Week 0}]-[\text{Week 23}]}{23 \text{ weeks}}$). Table C-6 lists the rate of degradation for each compound by treatment. Rates equal to zero show that there was no change in concentration during the 23 week experiment. Compounds that show the same rate across each treatment indicate that there was no difference in effectiveness between treatments for that compound. Rates greater than zero represent concentrations of compounds that were decreasing during the test period. Rates less than zero represent concentrations of compounds that were increasing during the test period.

The randomized block / repeated measures method was used to determine if there was a difference between treatments. Calculations were accomplished using A Statistical Package for Business, Economics, and the Social Sciences (Dellen/MacMillan, 1992). The dependent variable was Rate. The factors were Compound, and Treatment. Table C-7 indicates the results of the analysis of variance, and Table C-8 show the results of the pairwise comparison of treatments.

Table C-6. Degradation rates for compounds monitored during the treatability study.

Compound	Degradation rate (µg/kg per week) for the following soil treatments				
	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5
Naphthalene[a]	0	0	0	0	0
Acenaphthylene[a]	0	0	0	0	0
Acenaphthene	22*	17*	22*	22*	22*
Fluorene	61*	57*	61*	61*	61*
Phenanthrene	1583*	1578*	1583*	1583*	1583*
Anthracene	139*	135*	139*	139*	139*
Fluoranthene	3683	3843	3796	2870	-261
Pyrene	174	261	478	348	217
Chrysene	391	652	778	774	683
Benzo(a)anthracene	87	426	539	539*	383
Benzo(b)fluoranthene	-43	43	217	87	87
Benzo(k)fluoranthene	74	91	148	113	170
Benzo(a)pyrene	48	52	74	48	78
Indeno(1,2,3-cd)pyrene[b]	-61	-65	-43	-65	-57
Benzo(g,h,i)Perylene	-57	-61	-30	-43	-43
Phenol[a]	0	0	0	0	0
2,4-Dimethylphenol[a]	0	0	0	0	0
Pentachlorophenol	10652	11565	11261	11522	10261

[a] = concentration began and ended below detection limit.
[b] = concentration began below detection limit but rose above detection limit by the end of the 23 week study.
* = concentration dropped below detection limit; actual degradation rate may be higher than indicated.

Table C-7. Randomized Block / Repeated Measures Analysis of Variance.

	SUM OF SQ'S	D.F.	MEAN SQ.	F(D.F./68)	P-VALUE
Compound	6.01614E8	17	3.53891E7	1225.18	1.34976E-77
Treatment	333845	4	83461.3	2.88945	0.0285648
Error	1.96417E6	68	28884.9		
Total	6.03912E8	89			

Estimated Mean = 996.087 Variance = 320.943 MSE(68) = 28884.9

Table C-8. Pairwise comparison of treatments.

Treatment Pair	VALUE	SD.ER.	T(68)	P-VALUE
Treatment 1 / Treatment 2	-102.416	56.6518	-1.80781	0.0750586
Treatment 1 / Treatment 3	-126.087	56.6518	-2.22565	0.0293565
Treatment 1 / Treatment 4	-115.218	56.6518	-2.03378	0.0458775
Treatment 1 / Treatment 5	16.6664	56.6518	0.294191	0.769508
Treatment 2 / Treatment 3	-23.6714	56.6518	-0.41784	0.677381
Treatment 2 / Treatment 4	-12.8019	56.6518	-0.225974	0.821899
Treatment 2 / Treatment 5	119.082	56.6518	2.102	0.0392597
Treatment 3 / Treatment 4	10.8695	56.6518	0.191865	0.848419
Treatment 3 / Treatment 5	142.754	56.6518	2.51984	0.0140946
Treatment 4 / Treatment 5	131.884	56.6518	2.32798	0.0228959

Appendix D

Numbers of Culturable Microorganisms in Treatment Soils Throughout the 23-Week Treatability Study, As Determined by Triplicate Plate Counts on 5% TSA Agar

Table D-1. Numbers of culturable microorganisms in five treatment soils.

Incubation time (wks)	Log_{10} CFU per gram of soil (wet wt)[a]				
	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5
0	4.1	6.7	7.7	5.3	4.0
2	7.9	8.2	8.0	7.9	7.7
7	7.6	7.9	7.7	7.8	6.9
10	7.6	7.8	7.6	7.8	6.5
15	7.6	7.9	7.6	7.6	6.8
20	7.1	7.8	7.7	7.6	7.0
23	7.1	8.0	7.6	7.6	6.7

[a]Average from triplicate spread plates; CFU = colony-forming units.

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Biographical Sketch

Timothy D. Hodge is a native of Hot Springs, Virginia. He received his Bachelor of Science in Biology from Virginia Commonwealth University in 1983. After college, he enlisted in the United States Army. He received his commission through Officer Candidate School in 1986. He has served in various military capacities to include company commander, and platoon leader.

After completion of his degree at Florida State University, the Army will assign Tim as an instructor in the department of chemistry at the United States Military Academy, West Point, New York, where he will teach chemistry and biology.